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Award Number: DAMD17-99-1-9092

TITLE: Cloning and Characteristics of Active Egr-1 Target Genes by In Vivo Crosslinking

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REPORT DATE: December 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2003	Annual Summary (1 May 1999 - 30 Nov 2003)

4. TITLE AND SUBTITLE

Cloning and Characteristics of Active Egr-1 Target Genes by In Vivo Crosslinking

5. FUNDING NUMBERS

DAMD17-99-1-9092

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

This project is designed toward the identification of a comprehensive set of target genes for the transcription factor Egr1. Since breast cancer cells often do not express Egr1 while normal breast epithelial cells do, it is important to define Egr1 target genes with the hope that critical patterns of Egr1 regulated gene expression active only in normal cells will be revealed. I have chosen to approach this project using an in vivo crosslinking and chromatin immunoprecipitation (ChIP), protocol.

Using this approach I have successfully cloned a newly identified Egr1 target gene called TOE1. This gene is currently being characterized, but has the property of an inhibitor of cellular growth when overexpressed. Furthermore, TOE1 may act through interaction and modification of the activity of p53.

I have furthered the search for Egr1 target genes using the ChIP approach by adapting an array hybridization protocol using custom generated mammalian gene promoter arrays using a high throughput approach

14. SUBJECT TERMS

Breast Cancer

15. NUMBER OF PAGES

29

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

This final report provides a detailed description of my research activities and accomplishments for the period of funding support from the USAMRMC Breast Cancer Research Program. The project had as its major goal to define important target genes for the Egr1 transcription factor in breast cells using the ChIP approach. With Egr1 being a primary response gene that is expressed in normal, but not rarely in breast cancer cells, identification of its gene targets represents an important means by which potentially important transcriptional deficiencies in breast cancer cells may be discovered.
Here, I present a detailed summary on this project and its accomplishments. I also attach three published manuscripts detailing the experimental protocols used for analysis of Egr1 target genes.

Body

The results highlighted from the first year documented the successful use of the *in vivo* crosslinking technique to recover DNA targets directly bound by a transcription factor, in this case Egr-1. Additionally, the application of multiplex PCR allowed the amplification of cDNA sequences permitting target gene identification and completing part one of the Statement of Work and initiating part two of the Statement of Work. These studies resulted in the publication of a BioTechniques article, attached in the appendix of this report.

The complex task of identifying target genes through which key Egr-1 regulatory effects result in the maintenance of normal breast cell function was addressed next.
After successfully multiplex amplification, it was critical to determine whether the protocol faithfully achieved the amplification of genuine Egr-1 target genes. To address this, I proceeded to isolate individual cDNA's to allow their characterization and sequence identification. During this process, I have sequenced a full length cDNA for which no homolog or ortholog is known, by genomic database searching. I have named this new gene TOE1 for Target of Egr-1. Therefore, to identify this new gene as a target for Egr-1 transcriptional activity, it was necessary to perform an in depth characterization of the gene. A full length cDNA was isolated and sequenced. While homology searches

of DNA databases revealed no match to the cDNA, I found that the human genome sequencing project was able to map the gene to human chromosome 1. In order to characterize and confirm the regulation of this new gene by Egr-1, I performed both RT-PCR and gel shift experiments. Following transfection of Egr-1 into H4 cells (which do not express endogenous Egr-1), a dose dependent induction of the expression of TOE1 was noted. This result indicated that Egr-1 was indeed able to regulate the expression of TOE1 by activating its transcription. We were also able to find that expression of TOE1 resulted in the inhibition of cell growth including a G2 cell cycle phase pausing, as well as an inhibition of transformed growth as determined by the ability of cells to form colonies. Experimental details of these studies is provided in the attached publication in the Journal of Biological Chemistry. These studies addressed, in part, the Statement of Work parts 3 and 4. Further studies on the mechanism by which TOE1 is able to inhibit cell growth were initiated. We have demonstrated that TOE1 can physically interact with p53 in a signal dependent manner and that this interaction can affect the transcriptional activity of p53 (Figures 1-2). We have also found that TOE1 can be phosphorylated in response to exposure to UV irradiation (Figure 3). We hypothesize that one potential activity of TOE1 is to serve as a modulator for transcription factors regulating growth and the cell cycle, and that this activity can be affected by signal transduction pathways resulting in the post-translational modification of TOE1.

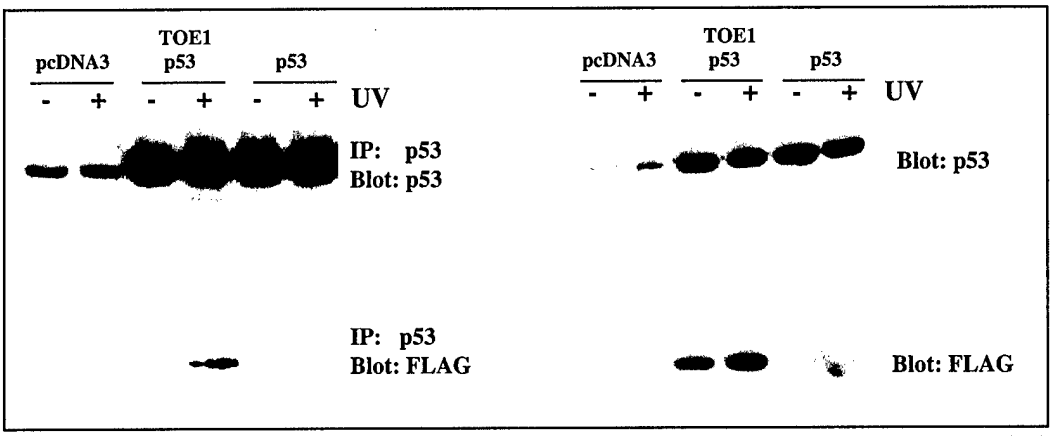

Figure 1. Co-immunoprecipitation of TOE1 with p53 following uv irradiation
293 cells were transfected with empty vector (pcDNA3), or vectors expressing p53 alone or together with FLAG-tagged TOE1. Following transfection cells were either untreated or exposed to 40 J/m^2 uv and incubated for 2h at 37 ^{0}C. Cell lysates were immunoprecipitated with anti-p53 antibodies, and western blots probed for either p53 or FLAG-TOE1. The left panels show the immunoprecipitates and the right panels show the presence of the transfected and expressed proteins from whole cell lysates.

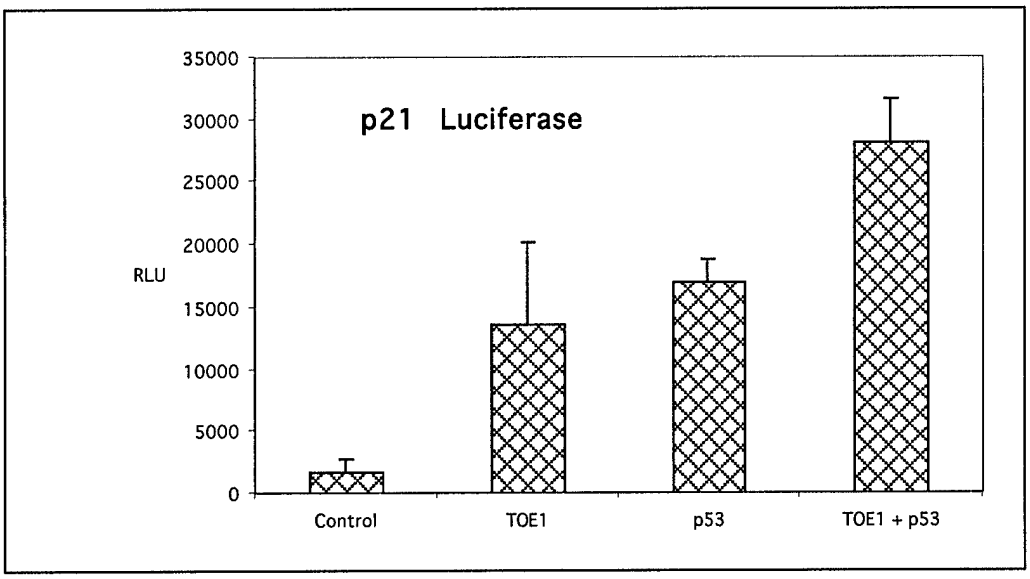

Figure 2. Transactivation of the p21 promoter by p53 and TOE1
293 cells were transfected with a luciferase reporter vector containing the p21 promoter together with the indicated expression vectors. Control samples were transfected with empty expression vector. 24 hours after transfection, cells were collected and assayed for luciferase activity by luminometry. Results are presented as the average of tripicate samples with the standard deviations indicated with error bars.

A further contribution to Statement of Work parts 2 and 4 were achieved by our findings that Egr1 serves as a direct activator of the recognized tumor suppressor gene PTEN. These studies show that Egr1 can activate PTEN expression during irradiation induced signaling in various cell systems including breast cells. These studies were published in Nature Cell Biology, and experimental details of this work can be found in the attached manuscript. Together, our work has demonstrated that chromatin immunoprecipitation can be an effective technique for the identification of transcription factor target genes, and that we have been successful in determining some relevant Egr1 targets for growth regulation.

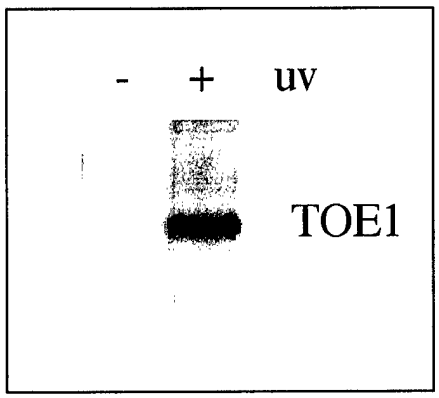

Figure 3. TOE1 is a phosphoprotein.
HT1080 cells were transfected with a TOE1 expression vector, and 24 hours later one dish of cells was exposed to 40J/m^2 uv irradiation while a second dish left untreated. The cells were labeled with ^{32}P inorganic phoshate for 2 hours and then extracted and immunoprecipitated with TOE1 antibodies. Phosphorylated TOE1 is seen only following exposure to uv irradiation.

While this work has been effective in target gene identification as well as novel gene cloning, we have extended our search for Egr1 target genes through the generation of a novel type of DNA microarray. We have successfully amplified over 4,000 gene promoter regions which have been spotted on glass slides to generate a "promoter array". We are using these arrays to screen, by hybridization, for additional Egr1 targets captured by the chromatin immunoprecipitation protocol. While these studies are ongoing, I believe that these arrays will greatly facilitate the ease by which large scale target identification will be accomplished. The use of this type of array will also be applicable to target gene identification for other important transcription factors involved in the control of breast cell growth and differentiation.

Key Accomplishments

1. Demonstration that Egr1 and crosslinked genomic binding sites can be immunoprecipitated and cloned.
2. Cloning of a newly identified target gene for Egr1 called TOE1. Demonstration that TOE1 plays a role in the growth suppressive effects of Egr1
3. Demonstration that the growth suppressive effects of TOE1 may be mediated by its interaction with and modulation of the activity of p53.
4. Demonstration that TOE1 is phosphorylated in UV treated cells and that this is modification is a likely event in regulating the interaction between TOE1 and p53.
5. Demonstration that another recognized tumor suppressor, PTEN, is a direct target for Egr1, and also contributes to the effects of Egr1 on the growth of breast cells
6. Generation of a new promoter array tool for the high throuput screening for target genes by chromatin immunoprecipitation coupled with array hybridization.

Outcomes

Personnel:

Ian de Belle, Ph.D., principal investigator

Virginie de Thillot, M.Sc., research technician

Aleks Surzycki, B.S., research technician

Publications:

1. de Belle, I., Mercola, D., and Adamson E.D.

Method for cloning in vivo targets of the Egr1 transcription factor. BioTechniques 29: 162 (2000). (Attached).

2. Virolle, T., Adamson, E.D., Baron V., Birle, D., Mercola, D., Mustelin, T., and de Belle, I.

The Egr1 transcription factor directly activates PTEN during irradiation-induced signaling. Nature Cell Biology 3:1124 (2001). (Attached).

3. Birle,D., Bottini,N., Williams, S., Huynh, H., de Belle, I., Adamson, E.D., and Mustelin,T.

Negative feedback regulation of the tumor suppressor PTEN by phosphoinositide-induced serine phosphorylation. Journal of Immunology 169:286 (2002).

4. de Belle, I., Wu, J.-X., Sperandio,S., Mercola,D., and Adamson,E.D.

In vivo cloning and characterization of a new growth suppressor protein TOE1 as a direct target of Egr1. Journal of Biological Chemistry 278:14306 (2003). (Attached).

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 16, Issue of April 18, pp. 14306–14312, 2003

Printed in U.S.A.

In Vivo Cloning and Characterization of a New Growth Suppressor Protein TOE1 as a Direct Target Gene of Egr1*

Received for publication, October 14, 2002, and in revised form, January 31, 2003
Published, JBC Papers in Press, January 31, 2003, DOI 10.1074/jbc.M210502200

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Egr1, an immediate early transcription factor, responds to diverse stimuli and affects gene transcription to accomplish its biological effects. One important effect of Egr1 expression is to decrease the growth and tumorigenic potential of several tumor cell types. To identify important Egr1 target genes, we have adapted a methodology involving formaldehyde-induced protein-DNA cross-linking, chromatin immunoprecipitation, and multiplex PCR. Using this approach, we report the cloning of a new Egr1 target gene that is able to account, at least in part, for the growth inhibitory activity of Egr1. We have named this new protein TOE1 for target of *Egr1*.

A common feature associated with the expression of immediate-early genes is their rapid, transient response to a diverse variety of extracellular signals. We have been studying the properties of the early growth response gene, Egr1, which can be transcriptionally induced by a wide spectrum of stimuli including growth factors, cytokines, stresses, depolarizing stimuli, phorbol esters, vascular injury, and irradiation, both ionizing and nonionizing, in a rapid and transient manner with kinetics mirroring those of c-*fos* (1). We have previously presented evidence suggesting a role for Egr1 in suppressing tumor cell growth (2, 3). Specifically, we demonstrated that overexpression of Egr1 in transformed cells suppresses growth in soft agar as well as inhibits their tumor formation in nude mice. Furthermore, it was shown that the DNA-binding domain of Egr1 is necessary for its ability to suppress tumor formation, highlighting the importance of its transactivation of downstream genes in this process (4). Together these results indicate that transformed cells can be induced to revert to normal growth patterns following the re-expression of Egr1. These studies suggest that the loss of Egr1 may result in the loss of cellular homeostasis because of a deficit in Egr1-responsive genes and that this may play a pivotal role in tumorigenesis. Clearly, the identification of a genetic profile of Egr1-responsive genes would constitute a significant step in understanding the different activities associated with Egr1, including its role in cellular growth control. Over the past several years there have been numerous studies identifying various individual Egr1 target genes in diverse cell and tissue types. Reported Egr1 targets include TGF-β1,[1] platelet-derived growth factors A and B, basic fibroblast growth factor, tissue factor, interleukin 2, and CD44 to mention only a few (reviewed in Ref. 5). These studies have focused on the *in vitro* analysis of an individual target gene in a specific cell type under a defined set of experimental conditions. As a step toward a more complete understanding of the biological role for a transcription factor, it would be informative to be able to identify *in vivo* target genes.

Currently, few techniques are available to address this issue. Both differential display and subtractive hybridization analyses are aimed at isolating messages that are up- or down-regulated from pools of RNA isolated from cells or tissues either positive or negative for the gene in question. One clear drawback with both of these techniques is that they select for any RNA message that shows a change in expression pattern. Therefore, when screening for changes in gene expression induced by a transcription factor, these methods do not select purely for direct targets. Recently we and others have described a method for the direct isolation of protein-bound DNA involving *in vivo* chemical cross-linking using formaldehyde followed by immunoprecipitation from chromatin (ChIP). This method was successfully used in applications ranging from examining chromatin structures surrounding the polycomb group proteins during *Drosophila* development (6) and the identification of nuclear matrix attachment sites (7) to the isolation of DNA sequences bound by Egr1 (8). In addition, the same cross-linking method has been used to examine nucleosomal structure, transcription factor occupancy of promoter sites, regions of histone acetylation, and mapping of telomere silencing protein binding, illustrating its broad application utility (9–12). Recently, coupling the ChIP approach with hybridization to genomic or promoter region DNA microarrays has allowed a comprehensive characterization of *in vivo* transcription factor DNA binding patterns (13–16).

In this report we have extended ChIP technology, allowing gene discovery of Egr1 target genes by multiplex PCR. Moreover, we present the cloning of a newly identified gene, called *TOE1*, as an Egr1 target gene. We have characterized TOE1 as a cell growth inhibitor by altering the cell cycle through the induction of p21. Furthermore, we show that the increase in the p21 level is consistent with a mechanism involving TGF-β1.

MATERIALS AND METHODS

Cells, Transfection, Antibodies, and Growth Assays—Both the H4 clone derived from the human fibrosarcoma cell line HT1080 and the

* This work was supported by National Institutes of Health Grants CA67888 (to E. D. A.) and CA76173 (to D. M.), by University of California Grant TRDRP 9KT-0078 (to I. d. B.), by United States Army fellowship DAMD17-99-1-9092 (to I. d. B.), and by a grant from the American-Italian Cancer Foundation (to S. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Ian de Belle and Sabina Sperandio dedicate this manuscript to the memory of Ted and Marilyn Crain.

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[1] The abbreviations used are: TGF, transforming growth factor; ChIP, chromatin immunoprecipitation; RT, reverse transcriptase.

Egr1 stably transfected H4 subclone E9 have been previously described (4). 293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. All of the DNA transfections were performed using LipofectAMINE 2000 (Invitrogen), following the manufacturer's instructions. Antibodies against cdc2, phospho-cdc2(Y15), and phospho-p53(S15) were from Cell Signaling Technology. Antibodies against cyclin B1, p21, and p53 were from Santa Cruz Biotechnology. Anti-actinin and the M2 monoclonal anti-FLAG antibody were from Sigma. For cell growth assays 20×10^3 control and TOE1 expressing 293 selected and pooled clones were seeded into 96-well plates in triplicate. At the indicated times, cell growth was determined using the CellTiter Cell Proliferation Assay (Promega).

In Vivo Formaldehyde Cross-linking and Chromatin Immunoprecipitation—Cross-linking and chromatin immunoprecipitation were performed as previously described (6, 8). Briefly, the cells were grown in 150-mm plates to 80–90% confluence and then cross-linked by the addition of buffered formaldehyde to a final concentration of 1%. Following exposure to formaldehyde at room temperature for a period of 30 min, the cells were lysed by sonication and chromatin purified by centrifugation through a 5–8 M urea gradient in TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA). Purified chromatin was dialyzed against 10 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5% glycerol to remove the urea. Samples of 30–60 μg of chromatin were digested with 10 units of *Eco*RI overnight at 37 °C and then precleared by the addition of nonimmune rabbit serum and protein A-Sepharose beads. The precleared samples were immunoprecipitated with affinity purified anti-Egr1 antibodies and protein A-Sepharose beads (17). DNA fragments cross-linked and co-precipitating with Egr1 were purified and ligated to *Eco*RI linkers consisting of 5′-AATTCGAAGCTTGGATCCGAGCAG-3′ and 5′-CTGCTCGGATCCAAGCTTCG-3′. Following ligation, the products were amplified using the 20-mer as primer. Amplification conditions were 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 4 min for 30 cycles. For direct amplification of the ChIP samples, no linker ligation was performed, and direct amplification from the Egr1 immunoprecipitates was done using specific primers for TOE1 (see below), TGF-β, and cyclophilin. The TGF-β primers used were 5′-GGGCTGAAGGGACCCCCCTC-3′ and 5′-TCCTCGGCGACTCCTTCCTC-3′. The cyclophilin primers used were 5′-CTCCTTTGAGCTGTTTGCAG-3′ and 5′-CACCACATGCTTGCCATCC-3′.

Library Multiplex PCR and TOE1 cDNA Cloning—Following amplification of linker-ligated products as described above, the linkers were removed by *Eco*RI digestion, and the products were purified using a PCR product purification kit (Roche Molecular Biochemicals). Multiplex PCR was performed using 100 ng of PCR products as the 5′ primer mix and a T7 oligonucleotide as the 3′ primer, with 100 ng of an excised undifferentiated NT2 cell cDNA library (Stratagene). 30 cycles of hot start PCR were performed using the following parameters: 95 °C for 45 s, 55 °C for 30 s, and 72 °C for 4 min. A 2-kilobase pair band derived from the multiplex PCR was excised from the gel, eluted, cloned into the pCR3.1 TA cloning vector (Invitrogen), and sequenced. Data base homology searches were performed using the BLAST program. To confirm the full-length TOE1 cDNA, we performed 5′ rapid amplification of cDNA ends using the fetal brain Marathon-Ready cDNA kit (Clontech), following the manufacturer's instructions. The TOE1 specific primer used for 5′ rapid amplification of cDNA ends was 5′-GTGAGGGGTACAGCTTTGCC-3′. A FLAG-tagged TOE1 expression vector was generated by PCR using the following primers: 5′-CCGAAGCTTATGGATTACAAGGACGACGACGATAAGGCCGCCGACAGTGAC-3′ incorporating the FLAG epitope tag and 5′-CCGGAATTCTCAGCTACTGCCCCAA-3′. PCR was performed for 30 cycles of 95 °C for 45 s, 62 °C for 30 s, and 72 °C for 2 min. The PCR product was digested with *Hin*dIII/*Eco*RI and cloned into the same sites in pcDNA3. All of the constructs were sequence-confirmed.

Cloning of the TOE1 Proximal Promoter and Luciferase Assays—The proximal region of the TOE1 cDNA sequence was cloned from human genomic DNA using the Advantage-GC genomic PCR kit (Clontech). Primers used for PCR were 5′-GCCGGTACCCGCTCTTACACC-3′ and 5′-CCCGTTAACGACACCGCTCGT-3′. The PCR parameters used were 95 °C for 45 s, 60 °C for 30 s, and 72 °C for 1 min for a total of 30 cycles. This reaction generated a 580-bp product immediately 5′ of the initiation codon. The PCR product was digested with *Kpn*I and *Hpa*I and cloned into the *Kpn*I and *Sma*I sites of pGL3basic (Promega). 293 cells were transfected in 12-well plates with a total of 500 ng of DNA using LipofectAMINE 2000 (Invitrogen). Transfected DNA consisted of 200 ng of expression vector DNA, 200 ng of reporter DNA, and 100 ng of cytomegalovirus-β-galactosidase DNA for normalization. 24 h after transfection, the luciferase assays were performed as described (8).

Mutagenesis—To generate the TOE1 expression construct without the putative nuclear localization signal, QuikChange mutagenesis (Stratagene) was performed. The primers used were 5′-GCGGCAGAGGACGCTTTATTGAACCTA-3′ and 5′-TAGGTTCAATAAAGCGTCCTCTGCCGC-3′. Construction of the correct deletion was confirmed by sequencing.

Gel Shift—The gel shift assay was performed as previously described (8) using the 580-bp radiolabeled TOE1 promoter region described above and recombinant Egr1 protein.

Confocal Microscopy—Control and TOE1 expressing H4 cells were dually stained with rabbit anti-FLAG (Affinity Bioreagents) and mouse anti-nucleolin (Santa Cruz Biotechnology) antibodies. Secondary labeling was performed using fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) and Texas Red-conjugated goat anti-mouse IgG (Jackson Immunoresearch).

Flow Cytometry—The cells were harvested and fixed in 70% methanol and stored at −20 °C until all of the samples were collected. The cells were collected by centrifugation at 2000 × *g* for 3 min, and the cell pellets were suspended in phosphate-buffered saline, digested with RNase A, and stained with propidium iodide.

Northern Blotting—A human Multiple Tissue Northern blot (Clontech) was hybridized with a PCR-generated TOE1-specific ^{32}P-labeled probe using the primers 5′-AAGCGGCGACGGCGACGACG-3′ and 5′-GTGAGGGGTACAGCTTTGCC-3′ following the manufacturer's instructions.

RT-PCR—To detect TOE1 expression following Egr1 transfection, total RNA was harvested from transfected cells using Tri Reagent (Molecular Research Center). Following DNase I treatment, 2 μg of RNA was used for reverse transcription using Moloney murine leukemia virus reverse transcriptase (New England Biolabs). TOE1 expression was then assessed by PCR using the same primers described above for Northern probe preparation, and glyceraldehyde-3-phosphate dehydrogenase expression was determined as a loading control using the primers 5′-AACCATGAGAAGTATGACAAC-3′ and 5′-GTCATACCAGGAAATGAGCT-3′. Expression of the p21 gene was determined using the primers 5′-CTCAAATCGTCCAGCGACCTT-3′ and 5′-ACAGTCTAGGTGGAGAAACGGGA-3′. TGF-β1 expression was assessed using the primers 5′-GCCCTGGACACCAACTATTGCT-3′ and 5′-AGGCTCCAAATGTAGGGGCAGG-3′, and cyclophilin A was amplified using the primers 5′-CTCCTTTGAGCTGTTTGCAG-3′ and 5′-CACCACATGCTTGCCATCC-3′. PCR conditions were 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min for 25 cycles.

Real time PCR reactions were performed using the one-step RT-PCR SYBR green kit from Roche using a Roche Light Cycler instrument. Following the RT reaction for 30 min, the PCR conditions were 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s for 40 cycles. mRNA quantitation was performed by measuring cyclophilin mRNA levels against a standard curve measurement of cyclophilin mRNA from a control sample. The primers used are described above.

In Vitro Kinase Assay—*In vitro* phosphorylation was performed as described (18).

RESULTS

Cloning of TOE1—We have previously characterized a clone of HT1080 cells, called H4, as a cell line that does not express either basal or UV-induced Egr-1. We have also described a series of stable transfected Egr1 clones (19). We used the clone with the maximum expression of Egr1, termed E9, to isolate and identify *in vivo* Egr1 target genes. We performed formaldehyde cross-linking on untreated and UV-stimulated cells followed by chromatin immunoprecipitation as described earlier (8). Because it is generally accepted that Egr1-binding sites usually occur within the proximal promoter region of genes, our immunocaptured Egr1-bound sequences are likely to consist of predominantly promoter regions with extensions into the 5′-untranslated region and even into the coding region. To identify target gene sequences we performed multiplex PCR using our immunocaptured Egr1-bound DNA sequences as 5′ multiplex primers. As template we selected a cDNA library and used a T7 primer that anneals 3′ to all cDNAs permitting full-length cDNA amplification. Using DNA captured from E9 cell Egr1 immunoprecipitates, we found that multiplex PCR-amplified products only in the presence of the multiplex primers, cDNA library, and the 3′ T7 primer (Fig. 1*A*, *lane 2*). When multiplex primers derived from UV-treated E9 cells were used, on occa-

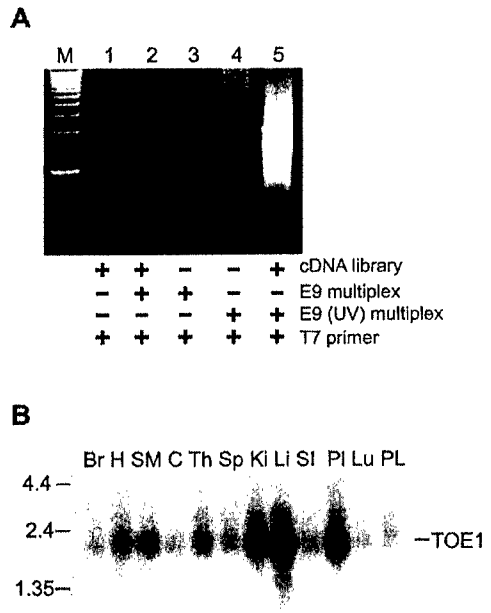

FIG. 1. **Cloning and characterization of the newly identified Egr1 target gene *TOE1*.** *A*, multiplex PCR amplification of Egr1 target genes from a NT2 cDNA library. Linker PCR amplification products of DNA from Egr1 containing immunoprecipitates were used as multiplex primers in a PCR reaction containing a NT2 cell cDNA library template as well as the T7 3′ primer. cDNA amplification products are seen in *lanes 2* and *5*, where all components are present. Both control and UV-treated E9 cells produced PCR products. *Lane M*, 1-kilobase pair DNA markers. *B*, multiple tissue Northern blot hybridized with a *TOE1* probe shows expression of an approximate 2-kb message in adult human tissues. The nucleotide sizes are indicated to the *left*. *Br*, brain; *H*, heart; *Sm*, skeletal muscle; *C*, colon; *Th*, thymus; *Sp*, spleen; *Ki*, kidney; *Li*, liver; *SI*, small intestine; *Pl*, placenta; *Lu*, lung; *PL*, peripheral leukocytes.

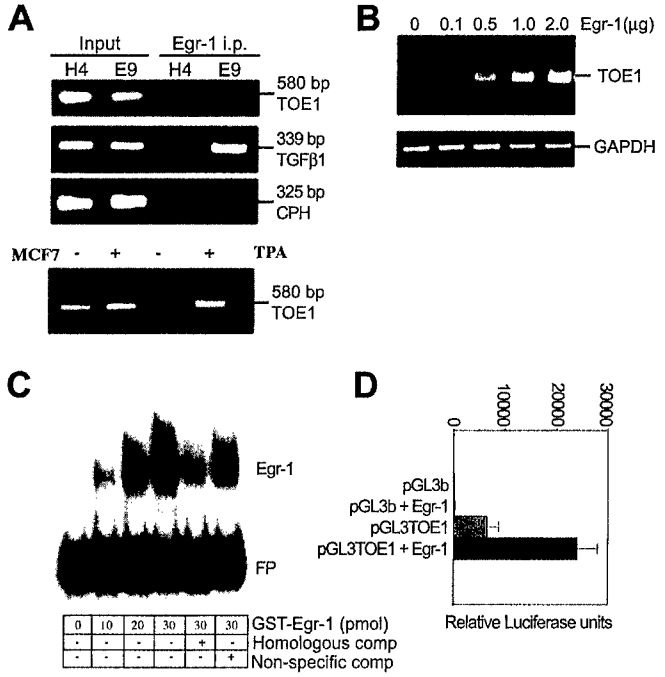

FIG. 2. **Egr1 binds to the 5′ region of *TOE1* and activates its expression.** *A*, PCR amplification of the *TOE1* 5′ region from cross-linked chromatin. Either total cross-linked chromatin (*Input*) or Egr1 immunoprecipitates (*Egr1 i.p.*) were screened directly for the presence of *TOE1* 5′ sequences by PCR using primers designed to amplify a 580-bp fragment 5′ of the initiation codon. The same samples were also used for amplifications using primers for TGF-β1 and cyclophilin A. The same primers were used to analyze Egr1 immunoprecipitates from untreated or 12-*O*-tetradecanoylphorbol-13-acetate-treated MCF7 cells. *B*, Egr1 expression activates *TOE1* expression. RT-PCR amplification of TOE1 from Egr1 transfected H4 cells. Increasing amounts of Egr1 (shown above the lane) were transfected into H4 cells, and total RNA was prepared 24 h later to perform RT-PCR for *TOE1*. Primers within the coding sequence of *TOE1* were designed to amplify a 454-bp product. An equal RNA loading in the RT-PCR reaction was determined using primers amplifying glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *C*, Egr1 binds directly to the TOE1 5′ region. The 580-bp region upstream of the initiation codon of *TOE1* was used as a probe in the gel shift assay. Increasing amounts of recombinant Egr1 showed the binding to this region. Specific binding was determined by adding either unlabeled homologous probe DNA or nonspecific DNA at a 50-fold molar excess. The positions of the free probe (*FP*) and Egr1 shift (*Egr1*) are indicated. *D*, Egr1 transactivates expression from the *TOE1* 5′ region. The same 580-bp 5′ sequence from TOE1 was cloned into the pGL3basic luciferase reporter. Empty reporter vector or the TOE1 reporter in the presence or absence of co-transfected Egr1 expression vector were transfected into 293 cells. 24 h later the cells were harvested and analyzed for luciferase activity. The results have been normalized for transfection efficiency as determined by β-galactosidase measurements. The results are plotted as the average values ± standard deviations. The experiment was repeated three times with similar results.

sion we found some self-amplification from the multiplex primers resulting in a high molecular weight smear (Fig. 1*A*, *lane 4*). However, the addition of cDNA library template produced a much stronger and distinctly different profile of amplified products (Fig. 1*A*, *lane 5*), suggesting that cDNAs were obtained from these primers as well. To directly address the question of whether these amplified cDNAs represented *bona fide Egr1* target genes, we isolated and cloned an individual target gene.

We focused on the distinct DNA band amplified using primers isolated from E9 cells and migrating with an approximate size of 2 kb (Fig. 1*A*, *lane 2*). Cloning and sequencing of this DNA revealed an open reading frame coding for a predicted polypeptide of 510 amino acids and with a predicted molecular mass of ~58 kDa. To confirm that this clone represented a full-length cDNA, we performed 5′ rapid amplification of cDNA ends. Sequencing results confirmed that the captured sequence represented a full-length cDNA clone. A data base homology search of the DNA sequence identified the chromosomal map position on human chromosome 1 (1p34.1–35.3). Comparison of the sequence of this region of chromosome 1 to our cloned cDNA identified an 8 exon gene. BLAST homology searches (20) revealed no extended homology with any known protein. However, a potential single zinc finger was noted as well as a possible nuclear localization signal.

To show that the clone represented an expressed gene, a multiple tissue Northern blot was hybridized and showed intense hybridization to a 2-kb mRNA species in six of the 12 tissues with the highest level of expression in placenta, liver, and kidney (Fig. 1*B*). We cloned the open reading frame of the cDNA, together with a FLAG epitope tag, into a mammalian expression vector and transfected the construct into H4 cells. Western analysis of cells transfected with the FLAG-tagged expression vector and anti-FLAG antibodies showed that the expressed protein migrated on SDS-PAGE with a molecular mass of ~60 kDa, in close agreement with its predicted mass of 58 kDa (data not shown).

TOE1 Is a Target for Egr1 Binding and Transactivation—To confirm the specificity of Egr1 binding to *TOE1 in vivo*, DNA recovered from immunoprecipitates was PCR-amplified to detect the 5′ region of *TOE1*. As shown in Fig. 2*A* we were able to amplify *TOE1* from E9 but not from H4 immunoprecipitates. We did, however, confirm the presence of the *TOE1* gene in the total chromatin fraction, thus ruling out the formal possibility that the *TOE1* gene is deleted in H4 cells. Further, the known Egr1 target gene TGF-β was also amplified from E9 cells (21). The lack of amplification of cyclophilin sequence served as a negative control. This provided evidence that *TOE1* was indeed a target of Egr1 in these cells and that the immunoprecipitated

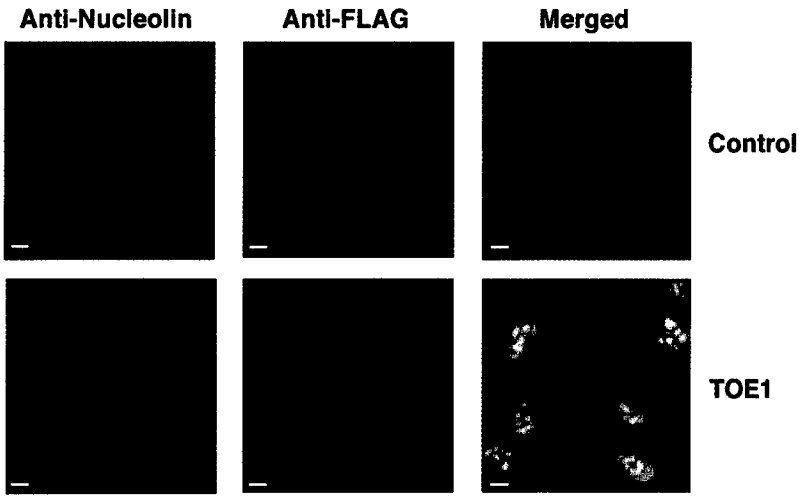

FIG. 3. **TOE1 is a nuclear/nucleolar protein.** Control vector and a FLAG-tagged *TOE1* expression vector were transfected into H4 cells. The cells were immunostained with antibodies to FLAG and to the nucleolar protein nucleolin. Texas Red and fluorescein isothiocyanate-labeled secondary antibodies were used to label nucleolin and FLAG, respectively. Confocal microscopy was performed showing nucleolar co-localization of TOE1 and nucleolin. The *bar* in each *panel* represents 10 microns.

DNA included the 5′ region of the gene. Because E9 cells constitutively overexpress Egr1, we sought to determine whether TOE1 is an Egr1 target in an alternate cell type upon transient Egr1 induction. MCF7 cells were stimulated to express Egr1 by 12-*O*-tetradecanoylphorbol-13-acetate treatment, and then the ChIP assay was performed on untreated or 12-*O*-tetradecanoylphorbol-13-acetate-treated cells. The results shown in Fig. 2*A*, TOE1 was also an Egr1 target gene in these cells. To determine the role of Egr1 in regulating the transcription of *TOE1*, we used RT-PCR following transfection with an increasing amount of an Egr1 expression vector and found a proportional increase in TOE1 expression (Fig. 2*B*).

Direct binding of Egr1 to the *TOE1* promoter region was assessed by a gel shift analysis using as probe a region spanning 580 bp upstream of the translation start. Using recombinant Egr1 we found specific binding to the probe (Fig. 2*C*). When oligonucleotides representing the consensus Egr1-binding site were used as competitor, effective competition was also observed (data not shown). As a test of the functional properties of the complex we inserted the same 580-bp 5′ region upstream of a luciferase reporter. We observed that this region responds to Egr1 expression by activating transcription (Fig. 2*D*). Together, these results are consistent with *in vivo* binding of Egr1 to and transactivation of the *TOE1* gene.

Subcellular Localization of TOE1—To determine the intracellular localization of TOE1, a FLAG-tagged expression construct was transfected into H4 cells. As shown in Fig. 3, following immunostaining for the FLAG epitope, the subcellular localization of TOE1 was distinctly nuclear. Transfection and staining of H4 and 293 cells (not shown) showed patterns of concentrated localization within the nucleus. These sites of concentration appeared to correspond to nucleoli. Dual staining using anti-FLAG and anti-nucleolin antibodies followed by confocal microscopy (Fig. 3) showed that most of the expressed TOE1 co-localized with nucleolin, indicating a predominant nucleolar location for TOE1. In addition to its nucleolar localization we observed intense staining for TOE1 as multiple nuclear speckles. As noted above, data base homology searches identified a putative nuclear localization sequence consisting of KRRRRRRREKRKR located at positions 335–347 in the 510-amino acid protein. Deleting the putative nuclear localization basic stretch of amino acids resulted in the cytoplasmic localization of TOE1 (Fig. 4), suggesting that this sequence is responsible for TOE1 nuclear targeting.

TOE1 Expression Affects the Growth of 293 and H4 Cells—To test whether TOE1 might be involved in mediating the growth effects of Egr1, we measured the growth rate of cells stably transfected with a TOE1 expression vector. Fig. 5*A* shows that

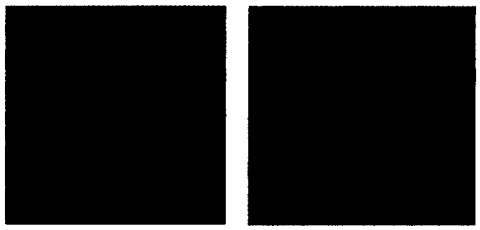

FIG. 4. **Identification of TOE1 nuclear localization sequence.** H4 cells were transfected with either a FLAG-tagged wild type TOE1 expression vector (*left panel*) or a FLAG-tagged TOE1 expression vector containing a deletion in the putative nuclear localization sequence (*right panel*). Following fixation, the cells were subjected to immunostaining using anti-FLAG (*red*). For the cells expressing the TOE1 nuclear localization sequence deletion, the nuclei were counterstained with 4′,6-diamidino-2-phenyl.

the growth rate of TOE1-expressing cells was severely reduced in comparison with empty vector control cells. The doubling time for control cells was ~24 h, whereas a pool of TOE1 expressing clones required 40 h to double in number. Transfection of the same vector expressing the calcium binding protein calbindin had no effect on cell growth (data not shown), suggesting that inhibition by TOE1 was not a nonspecific effect of protein over expression. Similar results were obtained in H4 cells (data not shown).

Cell growth inhibition in TOE1-expressing cells was also examined by performing colony forming assays. Control cells formed numerous rapidly growing colonies, whereas TOE1-expressing cells were only able to form 30% as many colonies (data not shown). To determine whether the decrease in cell growth of TOE1-expressing cells represented a generalized slowing of growth or a cell cycle stage-specific slowing, we performed flow cytometry on log phase cells. We found a significant increase in the fraction of cells present in the G_2/M phases of the cell cycle in TOE1-expressing cells (27%), compared with the control cells, with 13% of the cells in this fraction (Fig. 5*B*). We found no difference between the mitotic index of control and TOE1-expressing cells, suggesting that TOE1 was pausing the cells in the G_2 phase (data not shown). In addition, it should be noted that we found TOE1 expression to be highly influenced by the growth state of the cells. Specifically, we have found TOE1 expression to be regulated by cell culture density, possibly indicating a form of activation caused by contact inhibition.[2] The expression of TOE1 in dense cell cultures occurred even in cells that cannot express Egr1, indicating that although Egr1 can activate expression of TOE1, the

[2] I. de Belle and J.-X. Wu, unpublished observation.

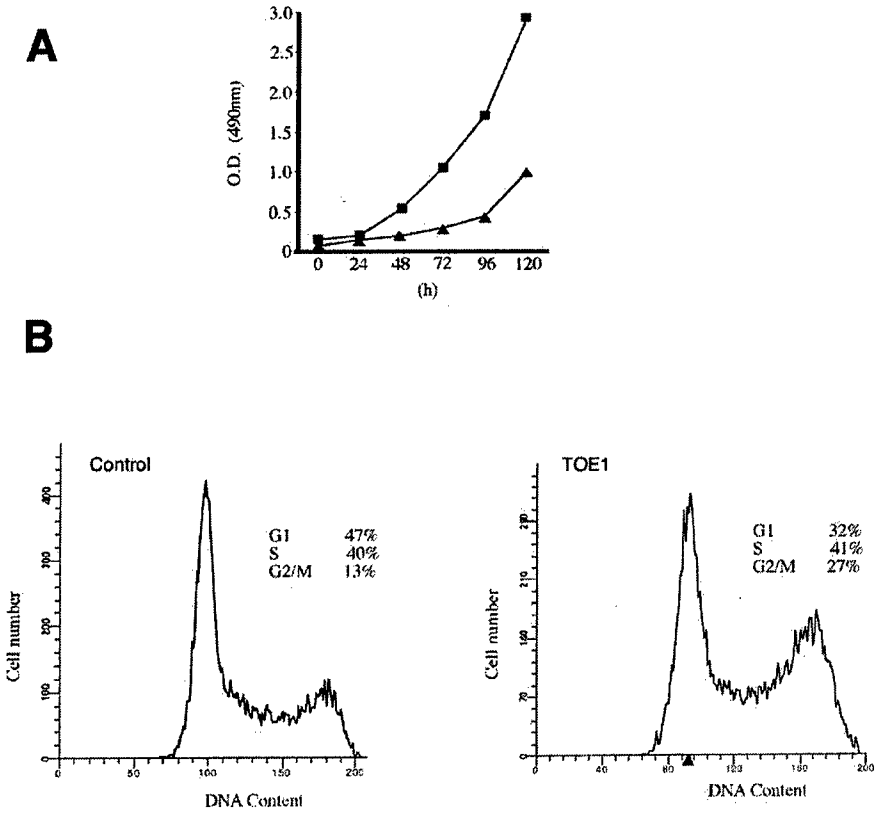

FIG. 5. **TOE1 expression affects cell growth and the cell cycle.** *A*, TOE1 decreases the growth rate of 293 cells. Pooled clones of empty vector or *TOE1*-expressing cells were used to determine their growth rate over a period of 5 days. *Solid squares*, control transfected cells; *solid triangles*, *TOE1*-expressing cells. The results are the averages of triplicate readings, and the experiment was repeated three times with similar results. *B*, *TOE1* expression affects the cell cycle. The cell cycle distribution of log phase growing control and TOE1 expressing clones of H4 cells was determined by flow cytometry. The calculated percentages of the cell cycle phases are indicated.

gene must be subject to additional forms of regulation.

TOE1 Causes an Increase in p21 Expression in H4 Cells—To investigate the mechanism of TOE1 induced G_2 phase delay, we performed Western blotting on several G_2 cell cycle markers. Fig. 6*A* shows that there was no significant change in cyclin B1, cdc2, or phospho-cdc2 levels between control, TOE1, and mutant TOE1-expressing cells (with the nuclear localization deleted). This suggested that the activation potential of the G_2-specific CDK complex was unaffected by the expression of TOE1. We therefore examined the possibility that the activity of the complex might be modulated by its known inhibitor p21. The level of p21 was dramatically up-regulated in TOE1-expressing cells but not in either control or TOE1 mutant cells. Because p53 is a known transactivator of the p21 gene, we examined the level and activation of p53 in our cells. We were unable to find a significant induction or activation of p53, at least insofar as serine 15 phosphorylation is concerned. Further exploration of the induction of p21 using RT-PCR showed that TOE1-expressing cells up-regulated p21 at the mRNA level (Fig. 6*B*). This activation was not seen in cells expressing non-nuclear mutant TOE1. To demonstrate that the increase in p21 was functionally associated with an effect on cdc2 activity, we immunoprecipitated cyclin B1 and measured the associated kinase activity *in vitro* with histone H1 as substrate. Fig. 6*C* shows a significant decrease in kinase activity only in TOE1-expressing cells, correlating with increased p21 expression in those cells.

Increased TGF-β1 in TOE1-expressing cells—Because Egr1 expression is known to affect TGF-β1 levels (21), we sought to determine whether the increase in p21 levels might be mediated by TGF-β1. Using real time quantitative PCR, we examined the TGF-β1 levels in cells transfected with a TOE1 expression vector. As shown in Fig. 7, using both MCF7 and H4 cells lines, we noted an increase in the level of TGF-β1 mRNA in TOE1 transfected cells compared with control transfected cells.

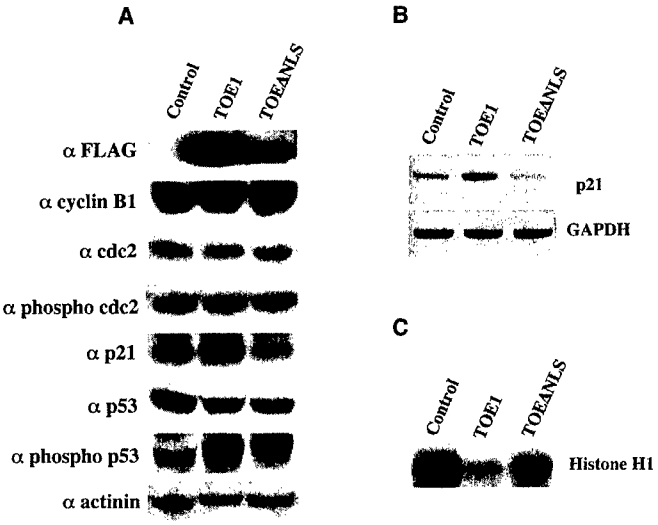

FIG. 6. **TOE1 affects growth inhibition through increased p21 expression.** *A*, control, TOE1, and TOE1ΔNLS cells were probed by Western blotting with the indicated antibodies. *B*, RNA was extracted from cells, and RT-PCR was performed for the expression of p21 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *C*, *in vitro* phosphorylation assay. Cyclin B1 immunoprecipitates were incubated with histone H1 and radiolabeled ATP. The products were visualized by SDS-PAGE and autoradiography.

DISCUSSION

With these studies we report, for the first time, the application of chromatin immunoprecipitation to cDNA cloning using a form of multiplex PCR. We have demonstrated that this technique was successful not only in cloning transcription factor target genes but also in the identification of a new target for Egr1. Together our results indicated that the multiplex amplification produced a genuine cDNA and that the cloned DNA represented an expressed gene. This newly cloned gene encodes

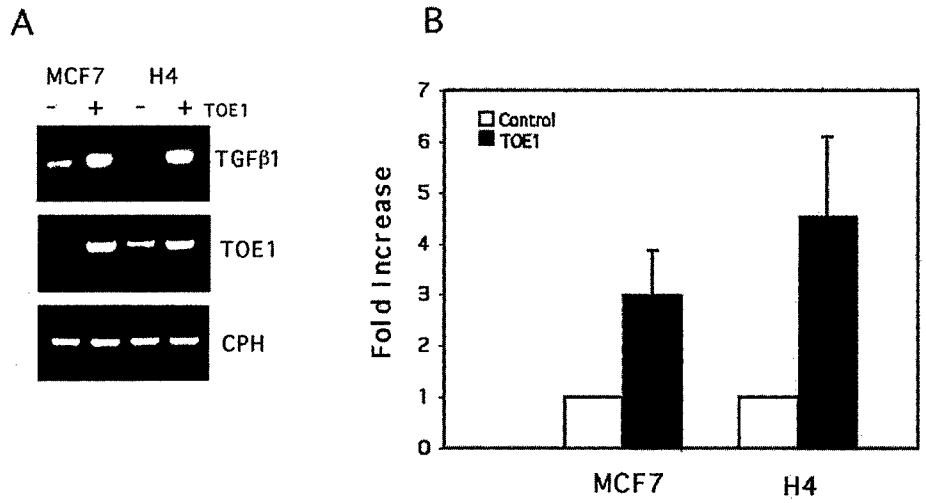

FIG. 7. **TOE1 expression affects the levels of TGF-β1 mRNA.** *A*, RT-PCR measurement of TGF-β1 and TOE1 from MCF7 and H4 cells transfected with an empty vector control (−) or a TOE1 expression vector (+). 24 h after transfection, RNA was harvested from the cells, and RT-PCR was performed using the protocol described under "Materials and Methods" with 25 cycles of amplification. Cyclophilin A amplification was used to demonstrate the equal RNA amounts included in each reaction. *B*, real time quantitative PCR was performed on MCF7 and H4 cells transfected with either control empty vector or a TOE1 expression vector. 24 h following transfection, RNA was collected and subjected to real time PCR for TGF-β1 mRNA quantitation. The *open bars* represent the relative quantity of TGF-β1 level in control cells, and the *closed bars* represent that for TOE1 transfected cells. mRNA samples were normalized to cyclophilin A levels. The results shown are the averages of four independent experiments showing standard deviations.

a 510-amino acid protein that we have shown to be an authentic Egr1 target gene. To confirm that the gene codes for an endogenously expressed protein, we have recently raised a polyclonal antibody using a synthetic peptide epitope derived from the predicted amino acid sequence. Preliminary testing has shown reactivity against both recombinant and an endogenous protein of identical molecular mass, suggesting that the cDNA is expressed at both the mRNA and protein level.

During the course of these studies an unpublished and unnamed cDNA generated through a library sequencing effort was deposited in the GenBank™ data base that was identical to our cloned cDNA (nucleotide accession number AK024011). Based on the sum of our observations, we have called this cDNA the HUGO approved name and symbol *TOE1* for target of *Egr1*. Expression of TOE1 was detected in all of the adult human tissues examined but at varying levels, indicating that the regulation of this gene may vary depending on cell or tissue type.

Examination of the sequence of TOE1 did not reveal conserved domain structures apart from a single potential zinc finger and a possible nuclear localization signal. Immunostaining confirmed that TOE1 was found localized to the nucleoplasm and nucleolus. Despite the absence of a recognized DNA-binding domain, we have examined the possibility that TOE1 might participate in transcriptional regulation. However, TOE1 cloned as a GAL4 fusion failed to activate a GAL4-binding site reporter, suggesting that TOE1 alone is not sufficient for transcriptional regulation. The possibility remains that TOE1 can participate in transcriptional regulation through protein interactions and indirect DNA association not recapitulated in the GAL4 fusion experiments. Although no extended homology to any known gene was noted by BLAST searches, a limited region of homology to poly(A)-specific deadenylation nuclease was revealed. We are currently investigating the possibility that TOE1 may function as a nuclease.

To better understand the biological role of TOE1, we examined the effects of its expression and noted a dramatic decrease in both the growth rate and colony growth of H4 cells. We found that this was not the result of a general decrease in growth rate but rather was due to a G_2 cell cycle phase delay. Furthermore, the G_2-specific cell cycle delay correlated with an increase in the expression of the cyclin-dependent kinase inhibitor p21. Deletion of the nuclear localization signal abrogated this effect, suggesting not only that TOE1 could induce cell cycle-specific G_2 pausing but also that its nuclear/nucleolar localization was critical for this function. The localization of TOE1 in the nucleolus may provide further evidence for a role in cell cycle regulation because it has been found that many important cell cycle proteins can be found in the nucleolus as a means of sequestration, thereby limiting their function until the appropriate time (22–24).

Because p21 is also able to inhibit cyclin-dependent kinase activities controlling passage through the G_1 restriction point, it would be predicted that the TOE1-directed increase in p21 levels would also display a G_1 phase pausing. Although we did not see this in log phase growing cells, when cells were synchronized in the M phase and then released to pass through G_1, we noted a marked delay in the TOE1-expressing cells (data not shown). This suggested that the increase in p21 levels was also active at the G_1 check point, but this was only seen if cells had been synchronized outside of the G_2 phase. Although p21 is well known for its activity in G_1 phase pausing, its role in G_2 is being increasingly recognized (25, 26). These results suggest that the mechanism by which TOE1 affects cell growth is through transcriptional up-regulation of the p21 gene. We have not, however, formally ruled out the possibility that the increase in p21 levels might be due to an increase in transcript stability rather than increased expression. Also, we have not completely ruled out a contributing role for p53 in the up-regulation of p21 but have demonstrated that p53 levels and serine 15 phosphorylation were not altered. Further, we have provided evidence that TOE1-dependent TGF-β1 activation may participate in the increase in p21. However, it also remains possible that TOE1 and p53 cooperate in the transactivation of p21 either directly or indirectly. We have preliminary evidence that TOE1 and p53 are able to interact physically, but the significance and specificity of this interaction remain to be analyzed.[3] Although the precise mechanism of action remains to be studied, our results have shown that expression of TOE1

[3] I. de Belle, unpublished observation.

leads to growth inhibition as well as a decrease in colony forming ability, likely involving the activation of p21. Given that these same features are seen following expression of Egr1, we expect that the downstream target TOE1 plays an important role in executing this physiological function of Egr1 in its proposed role as a tumor suppressor.

Finally, It is intriguing to note that the chromosomal location of *TOE1* maps to 1p34.1–35.3. Deletion of the distal portion of 1p accounts for a significant proportion of chromosome 1 aberrations and has been observed in brain, breast, ovarian, colorectal, and other tumor types (27–29). Combined data suggest that chromosome 1p likely harbors one and possibly multiple tumor suppressor genes, and given the growth inhibitory effect of TOE1, we are currently investigating the possibility that TOE1 may also function in this capacity.

Acknowledgment—We thank Dr. Erkki Ruoslahti for critical reading of the manuscript.

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APPENDIX 2

The Egr-1 transcription factor directly activates *PTEN* during irradiation-induced signalling

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Reprinted from Nature Cell Biology, Vol. 3 No. 12, December 2001

The Egr-1 transcription factor directly activates *PTEN* during irradiation-induced signalling

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The *PTEN* tumour suppressor[1] and pro-apoptotic[2] gene is frequently mutated in human cancers. We show that *PTEN* transcription is upregulated by Egr-1 after irradiation in wild-type, but not *egr-1*$^{-/-}$, mice *in vivo*. We found that Egr-1 specifically binds to the *PTEN* 5′ untranslated region, which contains a functional GCGGCGGCG Egr-1-binding site. Inducing Egr-1 by exposing cells to ultraviolet light upregulates expression of PTEN messenger RNA and protein, and leads to apoptosis. *egr-1*$^{-/-}$ cells, which cannot upregulate *PTEN* expression after irradiation, are resistant to ultraviolet-light-induced apoptosis. Therefore, Egr-1 can directly regulate *PTEN*, triggering the initial step in this apoptotic pathway. Loss of Egr-1 expression, which often occurs in human cancers, could deregulate the *PTEN* gene and contribute to the radiation resistance of some cancer cells.

The *PTEN/MMAC1/TEP1* tumour suppressor gene is mutated in a wide range of human cancers[3,4], and germline mutations of *PTEN* have been found in three inherited hamartoma tumour syndromes[5–7]. *PTEN* encodes a lipid phosphatase that removes the D3 phosphate from phosphatidylinositol-3-phosphate substrates, thereby inhibiting the generation of second messengers[8]. Deleting the *PTEN* gene in mice leads to embryonic lethality because of hyperproliferation of embryonic cells[9]. In Jurkat T leukaemia, breast and thyroid tumour cell lines, transient and inducible ectopic expression of PTEN induces cell-cycle arrest and cell death[10–12]. Expression of PTEN in PTEN-deficient multiple myeloma cells abolishes tumour growth *in vivo*[13]; PTEN phosphatase activity is essential for this effect[8]. Together, these findings suggest that loss of PTEN activity sensitizes cells to malignant transformation and imply that PTEN is a regulator of an important physiological pathway.

There have been no reports of transcriptional regulation of the *PTEN* gene to date, but a genomic fragment of DNA containing the full-length *PTEN* gene has been sequenced (K. Jensen *et al.*, unpublished results; GenBank accession number AF067844). We noticed that a 2-kilobase (kb) fragment upstream of the translation start site is highly GC-rich and contains several potential binding sites for the 'early growth response-1' (Egr-1) transcription factor; we tested these binding sites for activity. The hypothesis that Egr-1 (ref. 14) might regulate the transcription of the *PTEN* gene was attractive because the mutations in the two genes lead to some similar effects in cells, with variations depending on specific cell types. Like *PTEN*, the *Egr-1* gene also has growth-suppressing activities[15–19] and it was reasonable to think that *PTEN* could be regulated at least in part by Egr-1. A growing body of evidence indicates that Egr-1 is also required for apoptosis in some cells[20,21]. The results described here support the hypothesis that Egr-1 transactivates the *PTEN* gene to induce apoptosis.

Irradiation greatly upregulates production of Egr-1 (ref. 22). We used ultraviolet-C radiation to stimulate endogenous Egr-1 in 293T human fetal kidney and normal mouse mammary gland (NMuMG) epithelial cells to test whether *PTEN* expression was affected. Figure 1 shows that 4 h after irradiation with ultraviolet-C light at 40 J m^{-2} both PTEN and Egr-1 mRNAs (Fig. 1a, lanes 3 and 5) and protein (Fig. 1b, lane 2) were strongly upregulated, as was expression elicited by exogenous Egr-1 introduced by transient transfection (Fig. 1a, lane 2). This suggested that Egr-1 may act in the same signalling pathway as *PTEN*, and could be directly interacting with the *PTEN* promoter to effect this induction.

To find out how Egr-1 is able to regulate *PTEN* gene expression and what the mechanism might be, a 2-kb genomic DNA fragment corresponding to the *PTEN* promoter and its 5′ upstream regulatory sequences was amplified from human genomic DNA and cloned into a luciferase reporter plasmid thus creating *PTEN–luc*. This construct contained the full-length 5′ noncoding region of the mRNA. To determine whether the putative Egr-1-binding sites are involved in the regulation of the *PTEN* gene, *PTEN–luc* was transiently transfected into HEK293T, HT1080 fibrosarcoma (clone H4) or NMuMG cells with or without exogenous Egr-1 expression or with exposure to ultraviolet-C (40 J m^{-2}), γ-irradiation (5 Gy) or the DNA-damaging agent etoposide (20 μg ml^{-1}). Expression of *PTEN–luc* was well stimulated by exogenous Egr-1 expression, radiation or etoposide treatment in 293T (Fig. 1c) and NMuMG cells (Fig. 1d), but only ectopic Egr-1 was able to stimulate *PTEN–luc* expression in H4 cells, which lack Egr-1 (ref. 16; Fig. 1e). Using an antisense oligonucleotide to specifically inhibit Egr-1 expression blocked radiation- and etoposide-stimulated *PTEN* promoter activity (Fig. 1c, d). These results demonstrate not only that Egr-1 is able to stimulate *PTEN* promoter activity, but, more importantly, that Egr-1 is required for radiation- and etoposide-induced stimulation of *PTEN* expression. We also confirmed that the induction of *PTEN* promoter activity was dependent on the dose of Egr-1 transfected in 293T cells, which peaked at a fourfold induction (data not shown).

To determine whether *PTEN* transcriptional upregulation by Egr-1 occurs *in vivo*, wild-type and *egr-1*$^{-/-}$ mice were γ-irradiated and killed 2.5 h later, when Egr-1 induction is known to be high. Several tissues were rapidly dissected and flash frozen to analyse PTEN expression using reverse transcriptase–polymerase chain reaction (RT–PCR). Figure 1f shows that PTEN expression in wild-type mice was strongly increased in at least six tissues and moderately increased in three other tissues that have higher basal levels of

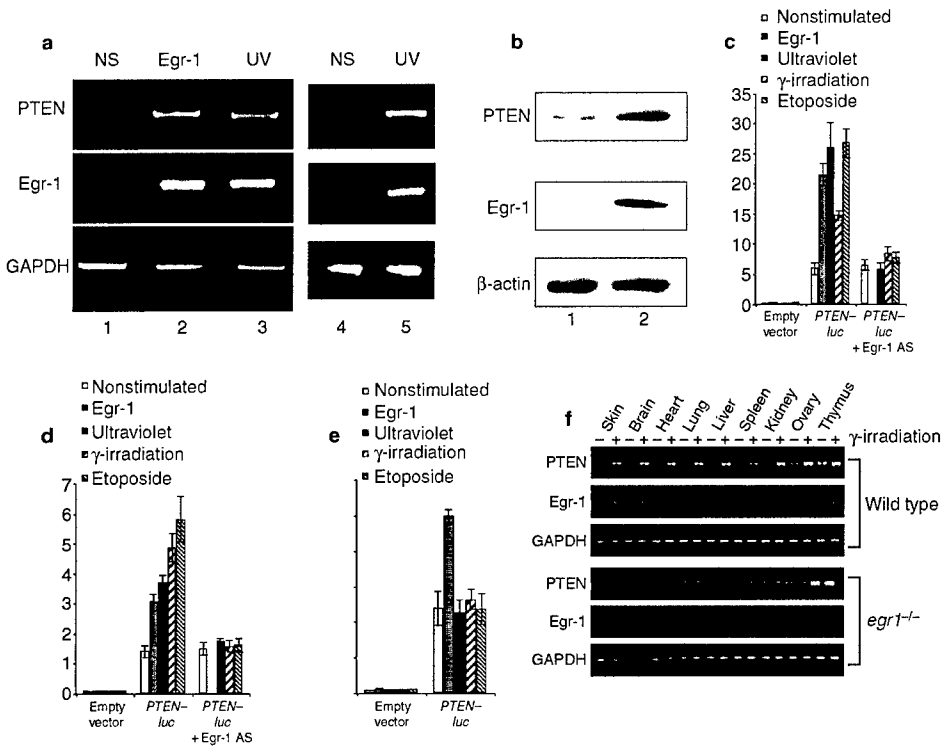

Figure 1 **Ultraviolet irradiation and ectopic Egr-1 stimulate expression of PTEN mRNA and protein. a**, Analysis of PTEN and Egr-1 mRNA expression levels by semi-quantitative RT-PCR assay with total RNA extracted from 293T nonstimulated (NS) cells as a template (lane 1); from 293T cells expressing exogenous Egr-1 (lane 2); ultraviolet-stimulated 293T cells (lane 3); nonstimulated mouse mammary cells.(lane 4); and ultraviolet-stimulated mouse mammary cells (lane 5). Amplification of GAPDH mRNA was monitored as an internal control. **b**, Immunoblot analysis for PTEN was performed with protein extract from nonstimulated (lane 1) and ultraviolet-stimulated NMuMG cells (lane 2), using a specific PTEN antibody as described in the Methods. Expression of β-actin was monitored as an internal control. **c–e**, The full-length *PTEN* 5′ promoter–reporter construct (*PTEN–luc*) was transiently transfected with or without 0.2 μM antisense (AS) Egr-1 oligonucleotides into 293T cells (**c**), NMuMG cells (**d**) or HT1080 cells (**e**), which do not express Egr-1, stimulated by ultraviolet irradiation, γ-irradiation, etoposide or exogenous Egr-1. Luciferase activity was determined as described in the Methods. Empty vector luciferase values correspond to the background expression of the pGL3 basic empty reporter gene. The luciferase activity values and error bars reflect the average and the standard deviation between at least three separate experiments with six replicates. **f**, Analysis of PTEN and Egr-1 mRNA expression levels by semi-quantitative RT-PCR, in nine different tissues from wild-type and *egr-1*$^{-/-}$ mice that were or were not γ-irradiated with 5 Gy and killed 2.5 h later.

PTEN mRNA. Corresponding with the radiation-induced PTEN, the expression of Egr-1 was induced about tenfold in most tissues (Fig. 1f, row 2). In contrast, Egr-1 expression in tissues from Egr-1-deficient mice was undetectable and uninducible (Fig. 1f, row 5) and PTEN expression was detectable but was uninduced by radiation (Fig. 1f, row 4). The analyses were performed with equal amounts of mRNA, as shown in the levels of the control mRNA from the GAPDH gene. These results indicate that Egr-1 is a major inducer of the *PTEN* gene *in vivo*.

The full-length *PTEN* promoter has numerous putative Egr-1-binding sites. To determine which sites might be responsible for Egr-1 stimulation, a series of deletions were made. The translation start site was designated as +1 for this study. The first three 5′ or distally truncated *PTEN* promoter constructs had similar stimulatory activity compared with the full-length promoter (data not shown), as did fragment Δ5′, which contained the proximal half of the sequences tested (−1 to −1031; Fig. 2a, b). Both transiently transfected Egr-1 and endogenous Egr-1 stimulated by ultraviolet irradiation of the cells produced very similar transactivating activity. Furthermore, the deletion of most of the 3′ end of these cloned regulatory sequences from the position −1 to −779 (the Δ3′ construct) did not abolish this stimulation. This narrowed the putative active binding sites to the remaining sequences located between nucleotides −779 and −1031, which includes the start of transcription. This fragment (*min PTEN–luc*) retains full activity (Fig. 2a, b). This fragment contained a 117-base pair (bp) GC-rich region containing three putative Egr-1-binding sites. Deleting this region in the context of the full-length *PTEN* regulatory sequences eliminated stimulation, narrowing the search to this short piece of DNA (Fig. 2a, b).

The three Egr-1-binding sites (EBSA, EBSB and EBSC) shown in Fig. 2c were individually mutated to give the mut A, mut B and mut C constructs. Mutation of EBSB and EBSC did not affect induction of the *PTEN* promoter in response to ultraviolet light or exogenous Egr-1 expression, whereas mutation of EBSA abolished the effect of both (Fig. 2d). These results demonstrate that the nine nucleotides GCGGCGGCG located between positions −947 and −939 constitute a functional *cis*-acting element necessary and sufficient for *PTEN* promoter stimulation by both transiently transfected Egr-1 and endogenous Egr-1 stimulated by ultraviolet irradiation.

The specificity of Egr-1 binding to the *PTEN* promoter was confirmed by *in vitro* gel-mobility shift assays using a 27-bp probe containing the normal or mutated EBSA site. Supershift assays with antibodies to Egr-1 confirmed the identity of Egr-1 binding to the probe (data not shown).

In order to assess whether direct binding of Egr-1 to the 117-bp portion of the endogenous *PTEN* regulatory sequences occurs in intact cells[23], we performed chromatin crosslinking studies on liv-

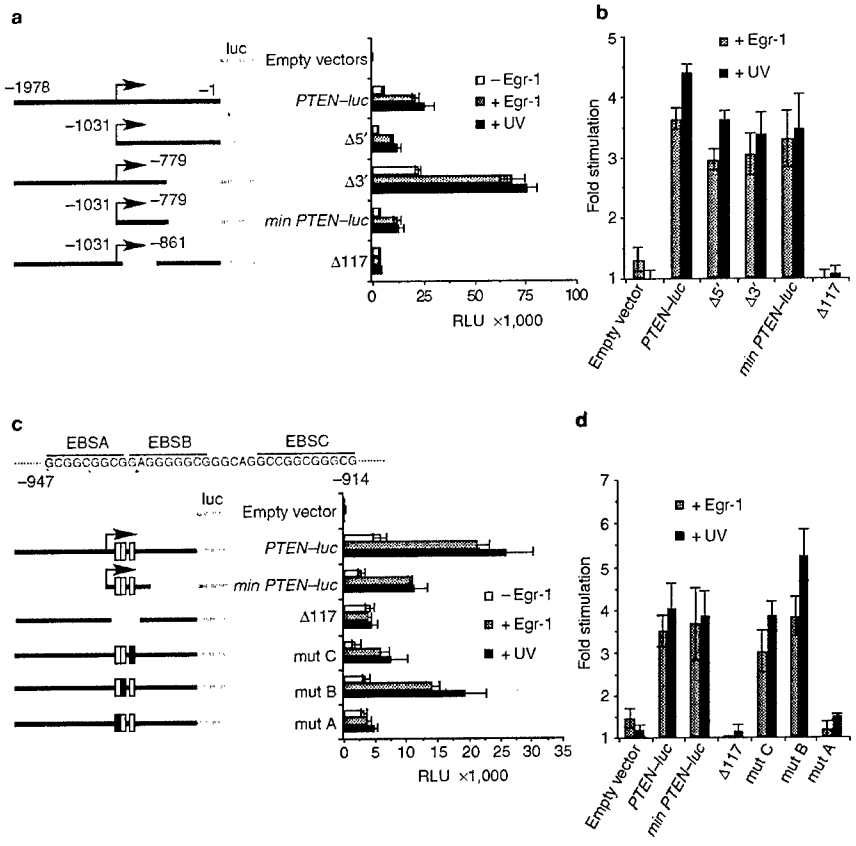

Figure 2 **Mapping the Egr-1-responsive element in the *PTEN* promoter.** **a**, Full-length and deleted *PTEN* regulatory sequences are represented on the left. The numbers indicate the position of the deleted fragments relative to the translation start site (+1). An arrow represents the start of transcription. Right, *PTEN–luc* and the deleted constructs were transiently transfected into 293T cells stimulated or not with ultraviolet irradiation or ectopic Egr-1 expression. Luciferase activities were assayed as described in the Methods and are shown as absolute values of relative luciferase units (RLU). **b**, Ultraviolet and Egr-1 stimulated activity. The values (x-fold stimulation) are derived from the ratio between the basal and the Egr-1-induced promoter activities shown in **a**. **c**, A GC-rich region in the *PTEN* 117-bp fragment corresponding to the three putative Egr-1-binding sites (EBSA, EBSB and EBSC). Left, unmutated EBSA, EBSB and EBSC are represented in the context of the full-length promoter by open boxes and mutated versions by black boxes. Right, the wild-type and mutated constructs were transfected into 293T cells stimulated or not by ultraviolet or exogenous Egr-1, and assayed as described in the Methods. **d**, Ultraviolet- and Egr-1-stimulated activity represented as the ratio (x-fold stimulation) between the basal and the Egr-1-induced absolute values of the promoter activities assessed in Fig. 2c.

ing cells, recovering Egr-1-binding sites by immunoprecipitation (see Methods). Three different conditions of 293T cells were tested: cells transfected with empty expression vector without ultraviolet irradiation was a control; a similar sample irradiated to induce endogenous Egr-1; and cells transfected with an Egr-1 expression vector. Egr-1 became fixed to its DNA target sequences after chromatin crosslinking, allowing recovery of the Egr-1-bound genomic DNA fragments by specific Egr-1 immunoprecipitation. We used a nonimmune serum as a negative control. The detection of the *PTEN* genomic fragment among all the captured fragments was made by PCR amplification using a pair of specific primers located at each end of the 117-bp sequence containing the Egr-1-binding site. Cells transfected with Egr-1 or ultraviolet-irradiated yielded an amplified product that showed the same migration pattern (Fig. 3, lanes 5, 6) as the 117-bp *PTEN–luc* control fragment (Fig. 3, lane 7). In contrast, no amplification was found either for the nonirradiated cells containing empty vector (Fig. 3, lane 4) or for the control nonimmune serum immunoprecipitated samples (Fig. 3, lanes 1–3). Each PCR band was purified and its identity verified by DNA sequence analysis. The sequences of the amplified PCR bands were identical to the 117-bp region in the *PTEN* promoter (data not

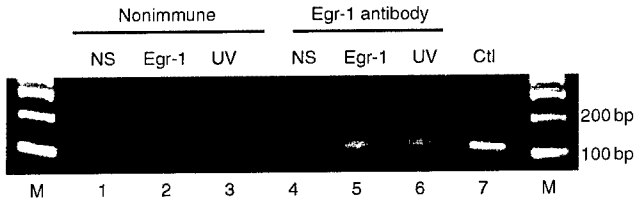

Figure 3 **Egr-1 binds directly to *PTEN* regulatory sequences *in vivo*.** Nonstimulated 293T cells (NS, lanes 1 and 4), 293T cells stimulated by Egr-1 (lanes 2 and 5) or ultraviolet light (lanes 3 and 6) were chromatin crosslinked and then immunoprecipitated[23] with a specific Egr-1 antibody or a nonimmune control antibody. The detection of the *PTEN* GC-rich 117-bp captured fragment was performed by PCR as described in Methods. Lane 7 corresponds to the control (Ctl) 117-bp *PTEN* fragment directly amplified from *PTEN–luc*. Lanes 1, 2 and 3 show the PCR amplification from the control nonimmune immunoprecipitation and lanes 4, 5 and 6 correspond to the *PTEN* 117-bp captured fragment amplified from the Egr-1-specific immunoprecipitation. M, marker.

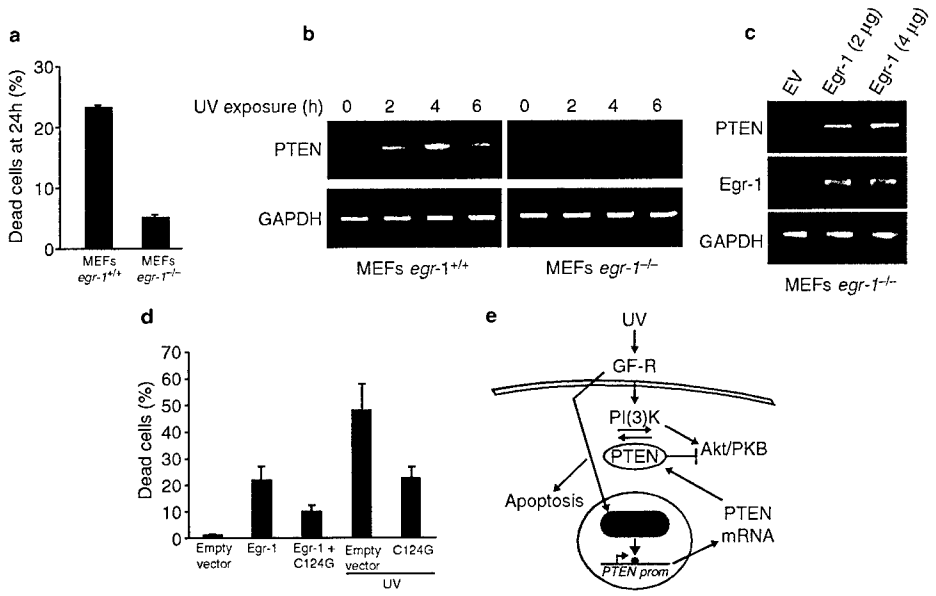

Figure 4 **Egr-1 is required for PTEN-dependent ultraviolet-induced apoptosis. a**, Monolayers of wild-type and Egr-1-null mouse embryo fibroblasts (MEFs) were exposed to ultraviolet-C radiation as described, and 24 h later the ratios of fragmented nuclei were compared (means of two experiments). **b**, MEFs collected at the indicated times after irradiation were assayed for PTEN mRNA levels by semi-quantitative RT-PCR. **c**, Dishes (60 mm) containing *egr-1*$^{-/-}$ MEFs were transfected with 2 or 4 μg Egr-1 expression vector or empty vector (EV) and 30 μl of lipofectamine. The day after transfection the cells were collected and PTEN mRNA levels were assayed by semi-quantitative RT-PCR. **d**, Inhibition of PTEN activity decreases the sensitivity of the cells to ultraviolet-induced cell death. 293T cells were transfected with the expression vectors shown in the absence of irradiation, or irradiated with or without overexpression of the catalytically inactive form of the PTEN protein (Cys 124→Gly). Empty vector was transfected as a negative control. Dead cells were determined 24 h later by trypan blue staining. Detached and trypsinized cells were pooled and incubated with 0.2% trypan blue. Cell death is shown as percentage of blue cells. **e**, The signalling events leading to apoptosis in 293T and NMuMG cells. The red circle represents the Egr-1-binding site; GF-R, growth factor receptor; PI3K, phosphoinositide 3-kinase; Akt/PKB, Protein kinase B.

shown), demonstrating that this sequence is an Egr-1 target in living cells. This result is consistent with the *in vitro* data and clearly demonstrates that Egr-1 binds directly to the genomic *PTEN* 5′ noncoding region in cells that have been ultraviolet irradiated, as well as in cells that overexpress exogenous Egr-1.

In 293T cells, the *PTEN* gene is intact, because exogenous Egr-1 or ultraviolet-C irradiation induce PTEN mRNA and protein, followed by apoptosis 24 h later. It has been shown that ultraviolet irradiation leads to apoptosis of 70% of *pten*$^{+/-}$ mouse embryo fibroblasts (MEFs), but *pten*$^{-/-}$ MEFs survived[24] indicating that the loss of PTEN activity probably causes resistance to ultraviolet-induced apoptosis. Therefore, we tested *egr-1*$^{-/-}$ MEFs in which ultraviolet irradiation does not induce PTEN mRNA (Fig. 4b) and found that these cells were also resistant to apoptosis induced by ultraviolet-C irradiation (Fig. 4a). The introduction of exogenous Egr-1 into the *egr-1*$^{-/-}$ MEFs restored the stimulation of *pten* gene expression (Fig. 4c). In wild-type MEFs, high levels of Egr-1 and PTEN mRNA expression were induced 2–4 h after irradiation (Fig. 4b), and a significant proportion of cells died within 24 h. Furthermore, inhibiting endogenous PTEN activity by overexpressing the catalytically inactive form of PTEN[10] (the Cys 124→Gly construct) leads to a 50% decrease of the sensitivity of 293T cells to ultraviolet-induced apoptosis mediated by Egr-1 (Fig. 4d). Because both the absence of Egr-1 and the inhibition of PTEN activity allow the cells to survive this death signal, we conclude that Egr-1 induction of *pten* is required for ultraviolet-induced apoptosis.

Therefore, the Egr-1 transcription factor directly transactivates the *PTEN* gene and is responsible, at least in part, for the apoptotic response after *PTEN* is induced by radiation or etoposide. Loss of Egr-1 may therefore contribute to radiation resistance of some cancer cells. □

Methods

Transfection, ultraviolet/γ-irradiation and etoposide treatment

Cells were seeded into 48-well plates at the density of 12,000 cells per well one day before transfection. The transfection was performed with the Fugene 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN). Typically 250 ng of total plasmid DNA (100 ng of reporter construct, 100 ng of Egr-1-expressing vector, and 50 ng of empty pcDNA3 vector) were mixed with 1 μl, 2 μl and 3 μl of Fugene 6 respectively for 293T, H4 and NMuMG cells. Cells were collected 24 h after transfection and luciferase activities were assessed using the Luciferase Assay System (Promega, Madison, WI) and an EG&G Berthold LB96P luminometer (PE Biosystems, Wellesley, MA). Cells were ultraviolet-C irradiated (40 J m^{-2}) in a Stratalinker (Stratagene, La Jolla, CA), γ-irradiated (5 Gy) or treated with 20 μg ml^{-1} etoposide to induce a response that includes high and transient induction of Egr-1 (ref. 25). After treatment the cells were incubated 4 h before harvesting.

To block Egr-1 expression we used a 20-mer-phosphorothioate analogue antisense oligonucleotide highly specific to Egr-1 (sequence available upon request).

MEFs derived from *egr-1*-null mice[26]

Dermal fibroblasts from skin explants from *egr-1*$^{-/-}$ and *egr-1*$^{+/+}$ mouse embryos were cultured as described previously[27] to derive 3T3-type cells. MEFs (56 population doublings) were seeded at 1×10^6 per 60 mm dish overnight, irradiated with ultraviolet C light at 40 J m^{-2} and harvested at 0, 2, 4 and 6 h for RT-PCR analysis of Egr-1 and PTEN (see below). Duplicate dishes containing 5×10^6 cells were cultured for 24 h after irradiation and apoptosis was estimated by staining with propidium iodide and counting the proportion of cells with fragmented nuclei.

Plasmid construction

We amplified 5′ *PTEN* regulatory sequences by PCR from genomic DNA using a pair of appropriate primers (5′-*KpnI*-GCCGGGTTTCACGCGGC-3′ and 5′-*Hin*dIII-GTCTGGGAGCCTGTGG-3′) located respectively at the position −1 and −1,978 from the ATG. The amplified product was purified and cloned into the *KpnI*/*Hin*dIII-digested pGL3 basic reporter gene to give the *PTEN–luc* construct. The Δ5′ construct was amplified by PCR from *PTEN–luc* using the primers 5′-*KpnI*-CCTCCCCTCGCC-CGGCGCGG-3′ and 5′-*Hin*dIII-GTCTGGGAGCCTGTGG-3′. The amplified product was purified and cloned into the *KpnI*/*Hin*dIII-digested pGL3 basic reporter gene.

The Δ3′, min *PTEN–luc*, Δ117, mut A, mut B and mut C constructs were made by directed mutagenesis according to the Quick-Change kit protocol (Stratagene). To create Δ3′ and Δ117, respectively 778 bp and 117 bp were deleted from *PTEN–luc* using the oligonucleotides 5′-GAGTTGAGCCGCT-GTGAGGCGAGGCAAGCTTGGCATTCCGGTACTGTTGG-3′ and 5′-CTCGGTCTTCCGAGGCGC-CCGGGCGCGGCAGGATACGCGCTCGGCGCTG-3′, respectively. To create the min *PTEN–luc* construct, 778 bp were deleted from Δ5′ using the oligonucleotide 5′-GAGTTGAGCCGCTGTGAGGC-GAGGCAAGCTTGGCATTCCGGTACTGTTGG-3′. To create mut A, mut B and mut C constructs, the *PTEN–luc* EBSA, EBSB and EBSC were changed into *Eco*RI restriction sites using respectively the

following oligonucleotides 5′ AGGCGCCCGGGCTCCCGGCGAATTCGCG-GAGGGGCGGGCAGGCCGGCGGGCGGTGATGT 3′, 5′-AGGCGCCCGGGCTCCCGGCGCG-GCGGCGAATTCATTCGGCAGGCCGGCGGGCGGTGATGT-3′ and 5′-GGCGCGGCGGCG GAGGGGCGGGCAGAATTCGAATTCGTGATGTGGCAGGACTCTTTATG-3′. All the DNA plasmid constructs were purified using columns from Qiagen (Valencia, CA). The C124G catalytically inactive form of PTEN was made as described elsewhere[10].

RT-PCR and western blots

Both total RNA and proteins were purified from 293T cells using the TRIzol reagent (Life Technologies) according to the manufacturer's protocol. When the cells were ultraviolet irradiated, the extractions were done 4 h after treatment. RT-PCR was performed with 200 ng of total RNA as template using the superscript RT-PCR kit reagent according to manufacturer's instructions (Life Technologies, Rockville, MD). PTEN, Egr-1 and control GAPDH mRNAs were reverse transcribed and amplified using the specific primers: 5′-GACAGCCATCATCAAAGAGA-3′ and 5′-TGACGGCTCCTC-TACTGTT 3′; 5′-CCCAGCTCATCAAA 3′ and 5′-CACCACACTTTTGTC-3′; 5′-AACCATGAGAAG-TATGACAAC-3′ and 5′-GTCATACCAGGAAATGAGCT-3′, respectively. The amplified products were resolved on a 2% agarose gel.

For the western blot analyses, after extraction, the proteins were boiled for 3 min and separated with 10% SDS polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to Immobilon P membranes (Millipore Corporation, Bedford, MA), blocked, reacted with primary antibodies at 1 $\mu g\,ml^{-1}$ and an anti rabbit secondary antibody conjugated to horseradish peroxidase for enhanced chemiluminescence detection of the signals (Amersham, Grand Island, NY).

Crosslinking and identification of Egr-1 bound to DNA

Live cells were treated with formaldehyde to crosslink chromatin complexes and Egr-1-containing fragments were recovered by immunoprecipitation as described elsewhere[23]. Identification of the captured *PTEN* 5′ regulatory sequences was performed by PCR analysis using the primers 5′-CTCGGTCTTCC-GAGGC-3′ and 5′-CCGAGCGCGTATCCTG-3′. Two consecutive rounds of 30 cycles each of PCR were performed, using the captured fragments as templates. The bands were excised from the gel used to analyse the product, purified and sequenced using an ABI 377 sequencer system (ABI, Foster City, CA).

RECEIVED 21 JUNE 2001; REVISED 20 AUGUST 2001; ACCEPTED 18 SEPTEMBER 2001; PUBLISHED 6 NOVEMBER 2001.

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ACKNOWLEDGEMENTS
We are indebted to A. Krones for her skilled assistance in providing mutant and wild-type mouse tissues. We thank E. Ruoslahti and J. Reed for comments on the manuscript. We are indebted to J. Milbrandt, Washington University Medical School, St Louis, for the gift of Egr-1 knockout animals. This work was supported by grants from the National Institutes of Health (E.D.A., D.M. and T.M.); from the Department of Defense (E.D.A. and I. de B.) and from the Tobacco Related Diseases Research Program of the University of California (I. de B.).
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Research Report

Method for Cloning In Vivo Targets of the Egr-1 Transcription Factor

BioTechniques 29:162-169 (July 2000)

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ABSTRACT

A methodology is described that allows the in vivo trapping of transcription factors to their target regulatory elements in multiple genes simultaneously. Cross-linking using formaldehyde is the first of several steps to isolate, purify, clone and characterize multiple gene promoter DNA fragments. The example that we use indicates that the TGFβ1 gene is a direct target induced by Egr-1 in HT1080 cells that express constitutive Egr-1, thus explaining the growth retardation that follows Egr-1 expression. The genes identified using this procedure reflect the specific activities of Egr-1 at that moment in the cell and provide a method for confirmation of genes that are the direct targets of Egr-1 action.

INTRODUCTION

Egr-1 is an immediate early growth response gene induced in response to serum stimulation of quiescent or growing cells. The alternate names for Egr-1 reflect the mode of its discovery. We first reported the cloning of the Egr-1 gene in fibroblasts and its role in development and in neuronal activity and differentiation (16). Other names are Zif268 (2), indicating its zinc finger DNA binding motif; Krox24 (1), showing its relation to the Krüppel family of homeobox transcription factors; NGFIA (14), because of its induction in PC12 cells by nerve growth factor; and TIS8 (12), because it is a gene induced by phorbol ester tumor promoters. Egr-1 is also the most responsive (mRNA induced within 5 min, protein level maximal at 60 min) of the stress-activated genes. Egr-1 is induced by radiation at all wavelengths, all tested growth factors, calcium ions, reactive oxygen species, heavy metals, depolarization and stretch among other stimuli. Its actions are partly governed by the levels achieved, stability and state of phosphorylation. Egr-1 protein is generally short-lived with a half-life of 90 min, but its expression can be much extended after stress and certain other stimuli that lead to its phosphorylation (6).

The Egr-1 gene family codes for transcription factors that bind to GC-rich regions in gene promoters. There are several members with similar structural features, but only the zinc finger domain has high homology among family members. The DNA consensus binding sequence is GCGG/TGGGCG, and it occurs in the promoters of several growth-related genes including Egr-1 itself, WT1 (related to Egr-1), EGF receptor, PDGF-A, PDGF-B, FGF-2, Bcl-2, p53, TGFβ1, IGF-II, IGF-IR and a number of extracellular matrix genes. A notable feature of the GC-rich binding site for Egr-1 is that it occurs most frequently in the proximal promoter regions of many growth-related genes, especially those that do not have TATA or CAAT boxes.

One set of genes regulated by Egr-1 endows a tumor suppressor function, and another set endows a survival response to damaging irradiation. Another important function of Egr-1 concerns the differentiation of many cell types. The mechanism for a change in cellular responses caused by Egr-1 would be clarified if we knew what new promoters Egr-1 protein binds to affect the activities. This paper describes and tests such a method by applying a stimulus of UV-C irradiation to determine what new Egr-1 target genes are bound by Egr-1 in HT1080 human fibrosarcoma cells. The cross-linking of proteins to

DNA was achieved with formaldehyde and was based on a method described previously (5,15). The method is termed direct in vivo Egr-1 target (DIVET) cloning. The method may be applied to capture the target genes of any transcription factor for which there is a specific immunoprecipitating antibody.

MATERIALS AND METHODS

Cells and Culture Conditions

The human fibrosarcoma HT1080 cell clone, H4, was kindly provided by Dr. S. Frisch (The Burnham Institute). H4 cells stably expressing Egr-1, E9 cells, have been previously described (9). Cells were cultured in DMEM containing 10% FBS and maintained in a 5%-CO_2 atmosphere at 37°C.

Chemical Cross-Linking and Chromatin Isolation

Formaldehyde-induced cross-linking and chromatin isolation was performed as previously described (3,11). An outline of the procedure is shown as a flow diagram in Figure 1. The optimal time of exposure of H4 and E9 cells to formaldehyde was determined by pilot experiments to be 1–2 h. This length of exposure was found to give the best yield of Egr-1 capture from the cross-linked chromatin. Cross-linking was performed on approximately 1×10^7 attached cells in 15-cm dishes. Chromatin, purified by urea gradient centrifugation at 50 000× *g* for 16 h, was stored at -80°C. Before immunoprecipitation, 30–60 µg DNA in chromatin were digested overnight at 37°C with 60 U *Eco*RI.

Antibodies and Immunoprecipitation

An affinity-purified rabbit polyclonal antibody (8) raised against amino acids 27–318 was used for both immunoprecipitation of and Western analysis for Egr-1. The antiserum was affinity-purified over a column of recombinant Egr-1 coupled to CNBr-activated Sepharose® beads (Sigma, St. Louis, MO, USA).

For immunoprecipitation, 30–60 µg cross-linked and *Eco*RI-digested chromatin were brought to a volume of 1 mL in RIPA buffer to which 1 µg affinity-purified anti-Egr-1 antibody was added together with 40 µL 50% suspension of protein A-Sepharose beads. Samples were rotated overnight at 4°C, and the bead-captured immunecomplexes were washed four times with RIPA buffer. Samples were then washed with TE buffer [10 mM Tris (pH 8.0) and 1 mM EDTA] and divided into two equal aliquots.

For Western analysis, one aliquot was washed with 10 mM Tris (pH 7.5), 2 mM $MgCl_2$, then resuspended in 100 µL of the same buffer with 10 U DNase I (Promega, Madison, WI, USA) and incubated at 37°C for 10 min. The samples were then boiled in SDS sample buffer for 10 min and analyzed by 7.5% SDS-PAGE. Western analysis was performed using the same anti-Egr-1 affinity-purified antibody at a 1:1000 dilution.

For DNA recovery from the second aliquot, cross-links were reversed with heat and proteinase K (Promega) digestion and DNA purified as previously described (3). Using both proteinase K digestion and heat treatment has been shown to achieve total reversal of cross-linking, which yields completely protein-free DNA (15).

Linker Ligation and PCR Amplification of Captured DNA

The purified DNA following cross-link reversal was redigested with 20 U *Eco*RI at 37°C for 2 h to ensure all ends were compatible with the linkers. The digested DNA was ligated to *Eco*RI linkers consisting of the following oligonucleotides: 5′-AATTCGAAGCTTGGATCCGAGCAG-3′ and 5′-CTGCTCGGATCCAAGCTTCG-3′. Following linker ligation at 16°C overnight, the ligated DNA was subjected to PCR amplification.

PCR amplification of the linker-ligated DNA was performed using the 20-mer oligonucleotide from the linker ligation as the PCR primer and *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA), using buffer conditions supplied by the manufacturer. PCR cycling parameters were as follows: 95°C for 45 s, 65°C for 30 s and 72°C for 5 min. The number of cycles was determined empirically so that few or no PCR products were visible in the control samples consisting of Egr immunoprecipitates from H4 cells (Figure 3, lanes 1 and 2). The number of cycles performed was typically 20–30. PCR products were purified by agarose gel electrophoresis and QIAquick™ gel extraction (Qiagen, Valencia, CA, USA). Linkers were removed by *Eco*RI digestion, and products were ligated in *Eco*RI-digested

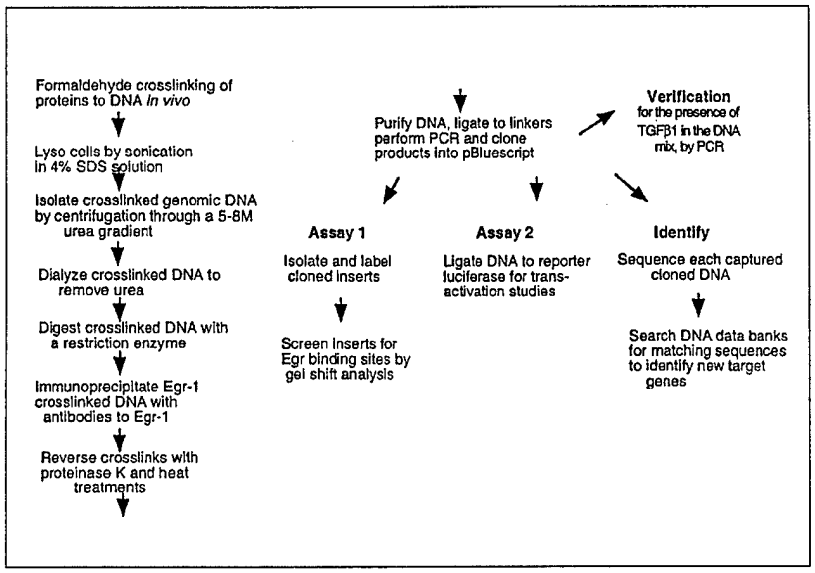

Figure 1. Flowchart to illustrate the early steps of the DIVET cloning procedure.

pBluescript® (Stratagene). XL2B cells (Stratagene) were transformed with the ligated products, and the individual bacterial colonies were isolated for characterization.

Gel Shift Assay

The inserts from individual colonies were isolated by *Eco*RI digestion and labeled for use as probes in a gel shift assay by fill-in synthesis using a Klenow fragment in the presence of [^{32}P]-dATP. Probes were purified by electrophoresis through a 6% acrylamide gel, elution in 10 mM Tris (pH 8.0), 50 mM NaCl and1 mM EDTA and then passed through an Elutip®-d minicolumn (Schleicher & Schuell, Keene, NH, USA). The gel shift assay was performed as previously described (7), using recombinant glutathione-S-transferase (GST)-Egr-1.

For gel shift competition, Egr-1 was allowed to bind to the probe for 15 min at room temperature, and then a 50-fold molar excess of unlabeled competitor oligonucleotide was added and samples incubated for a further 15 min. The wild-type Egr-1 binding sequence used as a specific competitor consisted of the following oligonucleotides: 5′-GATCACTCGCGGGGGCGAGGA-3′ and 5′-TGAGCGCCCCCGCTCCTCTTAG-3′. For a mutant Egr-1 binding site competitor, the following oligonucleotides were used: 5′-GATCACTCACATTTACAAGGA-3′ and 5′-TGAGTGTAAATGTTCCTCTAG-3′.

Luciferase Reporter Assay

Luciferase reporter plasmids were constructed by ligating the Egr-1-captured DNA sequences into pΔ56fos-Luciferase (a kind gift from Dr. C. Hauser, The Burnham Institute), which contains a c-*fos* minimal promoter upstream of the luciferase reporter gene. The reporter construct was transfected into H4 cells in the presence or absence of a cytomegalovirus (CMV)-Egr-1 expression vector by LIPOFECTAMINE™ transfection (Life Technologies, Rockville, MD, USA). Forty-eight hours after transfection, cells were collected, lysed in 100 mM KPO_4 buffer (pH 7.8) containing 0.2% Triton® X-100 and 1 mM dithiothreitol. The cell lysate was then assayed for luciferase activity in the presence of 10 mM ATP, 15 mM $MgCl_2$ and 0.1 mM luciferin substrate (Analytical Luminescence Laboratory, San Diego, CA, USA). Luciferase activity was measured in an EG&G Berthold microlumat LB96P luminometer (PE Biosystems, Foster City, CA, USA).

PCR Amplification of the TGFβ1 Promoter

Following immunocapture of Egr-1 cross-linked to its target sites from E9 cells, the purified DNA was subjected to PCR amplification reactions for the detection of the TGFβ1 promoter. The primers used for amplification were: 5′-GGGCTGAAGGGACCCCCCTC-3′ and 5′-TCCTCGGCGACTCCTTCCTC-3′. They represent base pairs -201 to -182 and +119 to +138 upstream and downstream, respectively, of the putative Egr-1 binding site in the TGFβ1 promoter (10,13). Amplification conditions were 30 cycles of 95°C for 45 s, 60°C for 30 s and 72°C for 45 s.

RESULTS

Immunocapture of Egr-1 from Cross-Linked Chromatin

An outline of our DIVET method to clone Egr-1 binding sites is presented as a flow diagram in Figure 1. Previously, we have shown that H4 cells do not express Egr-1, even following a stimulus known to induce Egr-1 expression. Importantly, we have found that H4 cells do not express Egr-1 following UV irradiation, normally a strong inducer of Egr-1. However, when H4 cells were stably transfected with Egr-1, several clones expressing Egr-1 robustly in a constitutive manner were isolated and one of these was named E9 (9). Since these cells provide an ideal comparison for the effects of Egr-1 expression, we chose for the present study to characterize the binding of Egr-1 to its in vivo sites in E9 cells. To assess the immunocapture of Egr-1 to its binding sites, we performed Western analysis of Egr-1 following immunoprecipitation of cross-linked and digested chromatin from both H4 and E9 cells. Because Egr-1 can be induced and activated by a variety of extracellular stimuli, we also examined Egr-1 immunoprecipitates from cells treated with 40 J/m^2 UV-C (254 nm) and incubated for 2 h following irradiation. Figure 2 shows the results of Egr-1 immunoprecipitation from samples of cross-linked chromatin. In H4 cells, there was little or no Egr-1 present in the immunoprecipitates, as we expected. In the Egr-1 constitutively expressing E9 cells, however, we successfully immunoprecipitated Egr-1 from both untreated and UV-C-treated cells. These results indicate that at least some of the Egr-1 expressed in E9 cells is bound to DNA in vivo and therefore has the potential to be functional. Furthermore, we observed that compared to untreated E9 cells, our immunoprecipitates contained considerably more Egr-1 following UV-C irradiation, which suggested that this stimulus promoted an increase in the binding of Egr-1 to its target sequences (Figure 2, compare lanes 3 and 4).

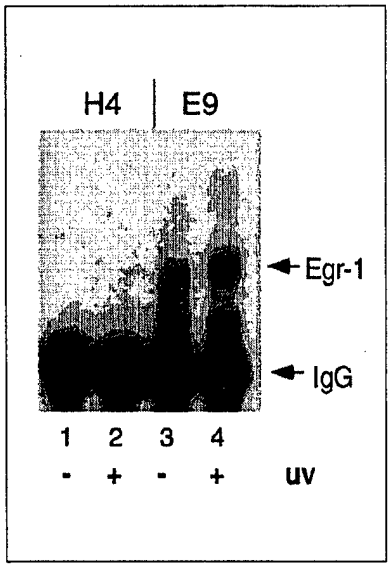

Figure 2. Egr-1 protein recovered from cross-linked chromatin. After immunoprecipitation of the chromatin fragments containing Egr-1, the DNA was digested, and the recovered protein was analyzed by SDS-PAGE and immunoblotting with the same affinity-purified rabbit anti-Egr-1 IgG. Lanes 1 and 2 show that little if any Egr-1 is recovered from H4 cells before and after UV irradiation. Lanes 3 and 4 show that Egr-1 recovered from E9 cells indicates a substantial increase in target frequency in UV-treated cells.

Amplification of Egr-1-Bound DNA

To assess the capture of Egr-1-bound DNA sequences, we performed linker ligation followed by PCR amplification on samples of Egr-1 immunoprecipitates from H4 and E9 cells. Figure 3 shows that after 20 cycles, DNA was amplified from E9 but not from H4 immunoprecipitiates. DNA fragments ranging in size from 0.5–3 kb were detected. This result is consistent with the presence shown by Western analysis of Egr-1 in E9 but not in H4 chromatin immunoprecipitates (Figure 2) and is a direct demonstration that Egr-1 is bound to DNA in these cells. Our results also suggest that following UV irradiation there may be additional sites to which Egr-1 binds, as evidenced by a different amplification profile seen in irradiated E9 cell (compare Figure 3, lanes 3 and 4).

The expression of Egr-1 in E9 cells is constitutive, and it is possible that the level is higher than one would expect after the normal induction of Egr-1. Therefore, we also tested physiologically induced Egr-1 expression in MCF7 human mammary carcinoma cells, using a two-hour exposure to tetradecanoylphorbol acetate (TPA). On a Western blot, the level of Egr-1 protein was twofold higher than that expressed in E9 cells, indicating that the method does not require high constitutive Egr-1 expression (data not shown). Also, multiple DNA fragments were captured using cross-linking of the Egr-1 to its target genes in MCF7 cells following our method. The sensitivity of the method is likely determined by the avidity of the antibody. To test the generality of the method, we also applied antibodies to c-Jun, with appropriate controls, with the result that presumptive c-Jun target genes were also captured after cross-linking (data not shown).

Specific Binding of Egr-1 to Captured and Cloned Binding Sites

To demonstrate that the DNA sequences that were amplified from E9 cells represent specific target binding sites for Egr-1, we performed gel shift assays on individual captured binding sites. Individual DNA sequences were isolated by ligating PCR products into a cloning vector and selecting single bacterial colonies after transformation, as described in the Materials and Methods section. Figure 4A presents gel shift results from three individually cloned and isolated Egr-1 binding sites from E9 cells. Purified recombinant Egr-1 bound to each of these Egr-1 binding sites. Egr-1 binding was abolished by competition using unlabeled wild-type consensus oligonucleotides but was unaffected by the mutant oligonucleotides, indicating that the binding was specific. The specific binding of Egr-1

demonstrated here confirms that our method is effective in selecting for DNA sequences to which a transcription factor, in this case Egr-1, is directly and specifically bound in the cell.

Captured Egr-1 Binding Sites Display Transcriptional Activity

An important criterion in the characterization of in vivo Egr-1 binding sites is to determine not only the specificity of Egr-1 binding but also that the functional consequence of binding. Because Egr-1 is an immediate early transcription factor, we examined whether or not the Egr-1 binding sites that we had captured and cloned were capable of influencing the transcription of a linked reporter gene. Individual Egr-1 binding sites derived from UV-treated E9 cells were cloned into a luciferase reporter vector and transfected into H4 cells alone or co-transfected with an Egr-1 expression construct. Figure 4B shows

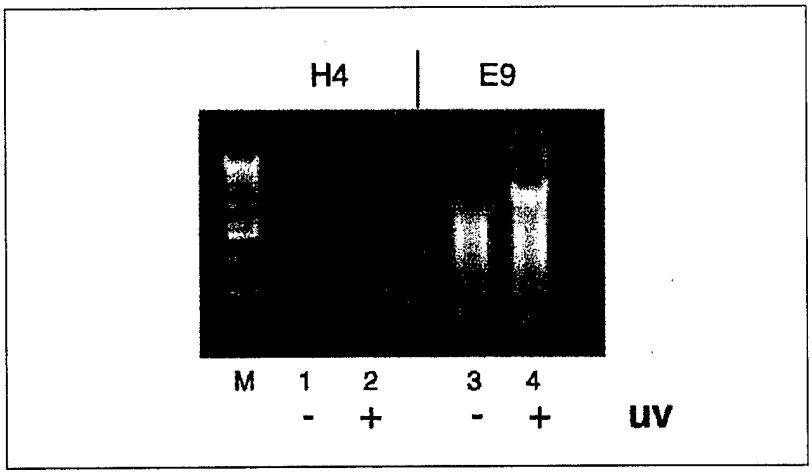

Figure 3. DNA fragments captured from HT1080 cells analyzed on an agarose gel. DNA fragments were amplified by PCR after reversal of cross-links between Egr-1 and its target DNA (see Materials and Methods for details). The lanes refer to the same cell extracts as in Figure 2.

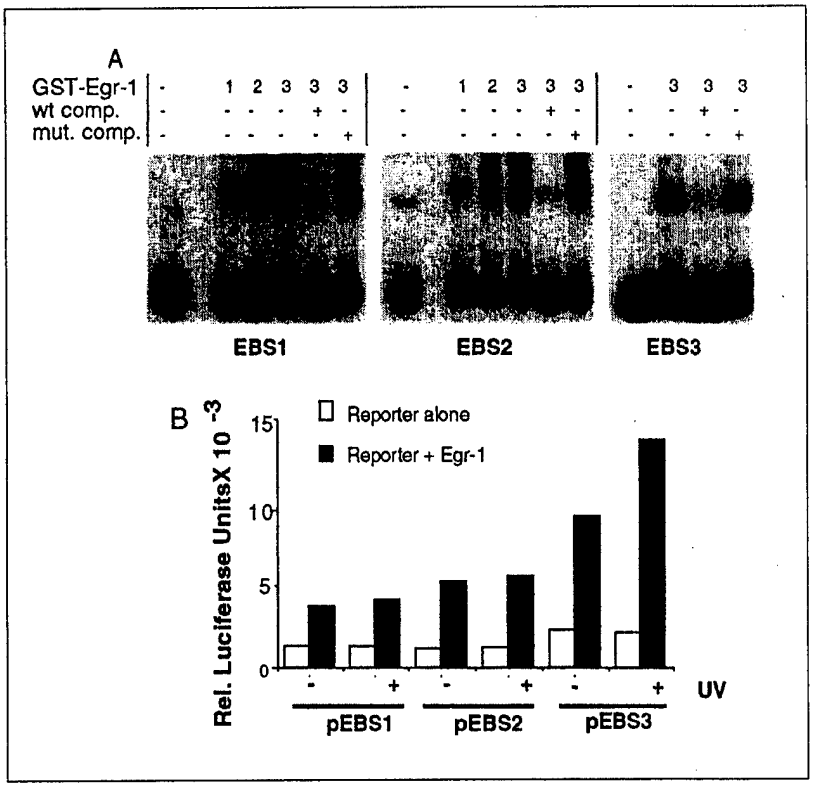

Figure 4. Egr-1 binding to and transactivation of captured DNA. (A) Gel Shift assays to show the binding specificity of captured DNA fragments. Three different DNA fragments were labeled and tested for putative Egr-1 binding sites (EBS). Recombinant GST-Egr-1 was allowed to bind as described in the Materials and Methods section. Unlabeled wild-type Egr-1 binding site oligonucleotides and mutated oligonucleotides were added to the indicated samples following Egr-1 binding as described in the Materials and Methods section. In all three cases, only the wild-type Egr-1 binding site oligonucleotides competed for the putative EBS. The concentration of Egr-1 used in gel shift studies, shown as 1, 2 and 3, corresponds to 50, 100 and 200 ng protein, respectively. (B) Transactivation assays with a luciferase reporter gene. The same captured DNAs as in A were ligated to a minimal promoter-luciferase gene. After transient transfection into H4 cells, the luciferase activity of cells co-transfected with Egr-1 (black bars) was induced compared to the "empty vector" control (white bars). Only EBS3 DNA contained a UV-activated target promoter.

the results from three Egr-1 binding sites and demonstrates that all binding sites were transcriptionally activated in the presence of Egr-1.

For Egr-1 binding sites 1 and 2, the transcriptional activation was modest and may reflect a requirement for additional cis and/or trans-acting factors for maximal activity. The transcriptional activity induced by binding sites 1 and 2 was not influenced by UV activation of Egr-1. In contrast, Egr-1 binding site 3 produced a significant trans-activation in the presence of Egr-1. Furthermore, following UV irradiation, we observed a further increase in transcriptional activity that was attributable to this binding site. This result provides a good example of the cloning of an Egr-1 binding site from irradiated cells that displays Egr-1-dependent and UV-activated transcriptional activity. From these results, it is clear that the Egr-1 binding sites captured by our method display both specificity of Egr-1 binding as well as Egr-1-dependent transcriptional activity. The method described here represents a useful means by which the direct binding targets of transcription factors can be cloned, identified and characterized.

Cross-Linking and Capture of Known Egr-1 Target Sites

Twelve DNA fragments were sequenced, and searches of GenBank® and other databanks indicated that none of the sequences matched known genes, expressed sequence tags or other DNA sequences. Of the 12 sequences, three were repeats and nine were unique. To confirm that our method of isolating Egr-1 target site sequences is working effectively, we set out to determine whether or not we were able to capture a known target of Egr-1. We have reported recently that, in E9 cells, Egr-1 is a direct transactivator of the TGFβ1 gene and contributes to the growth inhibition of these cells (13). We therefore tested for the presence of the TGFβ1 promoter sequence, containing the Egr-1 binding sites, in the DNA captured from E9 cells before and after UV irradiation. Figure 5 shows the amplification of the 338-bp sequence of the TGFβ1 promoter from E9 cells. Interestingly, we found the TGFβ1 promoter sequence bound by Egr-1 in E9 cells before but not after UV irradiation. Therefore, Egr-1 may no longer transactivate the promoter fragment 2 h after irradiation of the cells, indicating a change in function. These results validate our method of target site cloning and demonstrate the capability of cloning Egr-1 target sequences regulating a single-copy gene.

DISCUSSION

Egr-1 is an immediate early gene elicited by a large range of extracellular stimuli such as growth factors. Egr-1 has multiple activities in the cell by regulating the transcription of coordinated sets of genes. One of the central themes increasingly studied with transcription factor function is the search for sites within the genome that bind these proteins to endow their regulatory function. In the case of the immediate early transcription factors, it is also fundamental to understand the mechanism of binding site selection that allows the characteristic diversity of response. To identify the complement of sites to which a transcription factor binds in vivo at any moment and under a specific set of conditions would represent a significant step toward the understanding of the activities directed by that transcription factor.

We have described here our method for cloning, which was designed to allow these questions to be answered directly. Our results have demonstrated that Egr-1 is bound to numerous sites within the genome and has the potential to influence the transcriptional activity at these sites simultaneously. We have presented results on three cloned DNA sequences. Using this method, we have generated an Egr-1 binding site library that is being studied further.

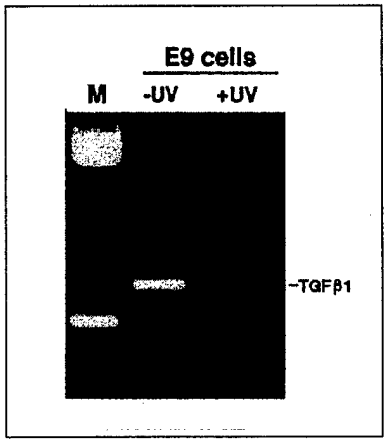

Figure 5. The capture of an authentic Egr-1-regulated promoter, the TGFβ1 promoter in the DNA targets from E9 cells proves that DIVET cloning is practical. Primers that bracket the putative Egr-1 binding site in the TGFβ1 promoter were used with the captured DNA as templates in a PCR. A band of the predicted size indicated that the TGFβ1 promoter was captured in the recovered cross-linked DNA.

It is possible that formaldehyde could cross-link protein to DNA nonspecifically, and it is likely that not all of the genuine binding sites are active in regulating transcription under all conditions. The cloning of transcription factor binding sites by this method must therefore include characterization for specificity of binding and a demonstration of functional activity associated with the binding of the transcription factor to the DNA. During the characterization of binding sites, some obvious considerations include the requirement for heterodimerization or cofactors. Dimerization has not been observed in the case of Egr-1, however. In many cases, a single transcription factor does not trans-regulate alone, but rather contributes to gene regulation as part of a complex set of protein-DNA and protein-protein interactions.

Despite these considerations, we have for the first time successfully cloned DNA sequences that both bind Egr-1 directly and influence transcription. In one case presented here, we have cloned a sequence that not only binds Egr-1 but is also UV responsive (Figure 4B, pEBS-3). This demonstrates the possibility of identifying specific targets of transcription factors in response to a specific stimulus. In another example, we showed that TGFβ1 gene promoter sequences were captured by cross-linking to Egr-1 in E9 cells (Figure 5). This not only proves the principle of the method (because TGFβ1 is a known target of Egr-1 induction) but also is the first demonstration that Egr-1 functions in vivo by its direct binding to the TGFβ1 promoter.

Our observation that Egr-1 was not bound to the TGFβ1 promoter following UV irradiation may indicate that, following this stimulus, there is a decrease in the Egr-1-induced growth suppression in these cells. This would be consistent with our recently published results showing that following UV treatment, the expression of Egr-1 is associated with an increase in cell survival that correlates with a transient increase in the rate of cell cycle progression (4).

Moreover, we are developing a further step in this cloning method by using the longest captured DNA frag-

ments as labeled probes to hybridize to multiplex arrays of cDNAs. Clearly, this technique has the potential for dissecting the diverse activities of transcription factors responding to a variety of signals and may reveal novel genetic targets of these factors. Moreover, the elucidation of gene clusters that are coregulated by a stimulus is a major advantage of this method.

ACKNOWLEDGMENTS

We gratefully acknowledge the support of grants from the Public Health Services, CA 67888 (E.D.A.) and CA 63783 and CA76173 (D.M.) from the National Cancer Institute and DAMD 17-99-9092 (I. de B.) from the Department of Defense.

Captured DNA sequences will be made available upon request.

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Received 10 December 1999; accepted 27 March 2000.

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